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THE EFFECT OF NOXIOUS AGENTS ON CREATINE, CREATININE, CHLORIDE AND WATER EXCRETION

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(Received 1 February 1939)

PREVIOUS observations [Selye, 1937*b*, 1938*a*] have shown that exposure to various non-specific noxious agents elicits a syndrome with characteristic morphological and chemical alterations. These changes are largely independent of the specific nature of the noxious agent used. They have been divided into stages, i.e. those of "alarm", "resistance" and "exhaustion".

Among the chemical changes characteristic of the "alarm" reaction are a decrease in the whole blood chlorides accompanied by a transitory increase followed by a decrease in blood sugar. In the stage of "resistance" both blood sugar and blood chlorides are above normal, while in the stage of "exhaustion" fatal hypoglycaemia may ensue which may or may not be accompanied by hypochloraemia [Selye, 1938*b*]. The haematocrit value is usually high and the blood volume low during the first and third stages, while during the second stage the haematocrit value tends to be below and the blood volume above normal [Selye, 1938*b*, 1938*c*; Selye & Foglia, 1938]. The effect on the excretion of ingested water has so far only been investigated in the first stage during which it was found to be subnormal [Howlett & Browne, 1937; Karady, Browne & Selye, 1938].

It seemed of interest, in view of the above-mentioned findings, to study the water and chloride during all three stages and at the same time to follow the creatine and creatinine excretion, especially in view of the fact that an increased urinary elimination of the latter metabolites is generally regarded as an indicator of increased cell decomposition and has frequently been observed under the influence of wasting diseases [Schittenhelm & Bühler, 1935; Gabrielli, 1935].

METHODS

Female rats of a "hooded" strain were used. They weighed 160–180 g. at the beginning of the experiment.

The effect on water excretion was measured by administering 10 c.c. of tap water by means of a stomach tube. The animal was placed in a metabolism cage and no food or water was given. Twenty-four hours after the administration of the tap water the amount of urine excreted was measured. The excretion of chlorides in the urine was measured by the method of Rusznyak [1920] and expressed as mg. chloride per 24 hr. The creatinine was determined by means of a modification of the Folin-Wu method using the Evelyn photoelectric colorimeter [Evelyn, 1936]. The creatine was determined by the usual method of conversion to creatinine and subtraction of the preformed from the total creatinine value. These results were also expressed as mg. excreted per 24 hr.

The noxious agents used were cold, muscular exercise and formaldehyde injections, as previous work had shown that all these agents were effective for the purpose in view. The animals exposed to cold were placed continuously for the whole length of the experiment in a refrigerator having a temperature of 2–5° C. They were also kept in the refrigerator during the period of urine collection. The group performing muscular exercise ran for a period of 1 hr. twice daily in drum cages (diam. 12 in., speed 18–20 rev. per min.). During the 24 hr. of urine collection the animals were not exercised. The group treated with formaldehyde received two subcutaneous injections of 0.5 c.c. of a 4% formaldehyde solution daily throughout the experiment.

RESULTS

The figures summarize the results obtained. Eight animals were used in each experimental group, except in the last two determinations in the formaldehyde group in which only six were used. The values given in the figures are average ones. The formaldehyde experiment was discontinued at the end of forty days owing to the development of cutaneous necroses.

The fact that treatment with all three stimuli did actually elicit the three-stage reaction expected was confirmed by autopsy in a few animals during the course of each experiment. This revealed, among other morphological signs, the formation of the very characteristic gastrointestinal ulcers and the presence of an enlarged adrenal cortex showing loss of lipid granules during the first and third stages while no such changes were seen during the second stage.

The zero columns in all the figures represent the average of ninety-five determinations on normal control animals given 10 c.c. of water by stomach tube and kept without food and water for 24 hr., but otherwise untreated. In all the experiments three normal controls were run with the treated groups each time a determination was done. It was found that repeating the procedure of giving water and collecting the urine on the same control animals had no significant effect on the results. These columns therefore represent the average of all the control animals

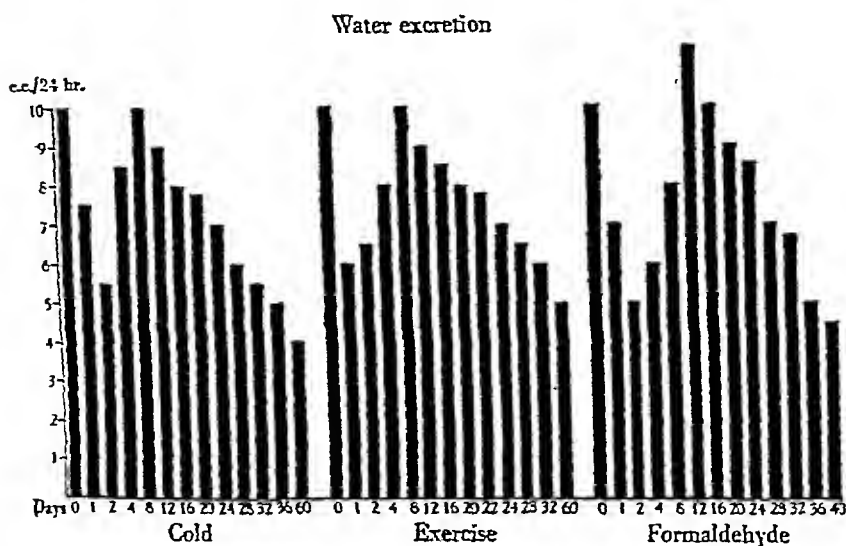


Fig. 1. The effect of exposure to cold and muscular exercise and of subcutaneous injections of formaldehyde upon the excretion of ingested water.

regardless of the experimental group with which they were run. In the control group all the ingested water is excreted at the end of 24 hr. 20 mg. is the average figure for chloride excretion, 7.6 mg. for total creatinine, of which 2.5 mg. is creatine.

Fig. 1 shows the effect of the three stimuli on the urine output. It will be seen that the excretion of water falls below normal at first, reaching its lowest point after 24-48 hr., as has been previously reported. Then it increases again, reaching a maximum between the 8th and 12th day of the experiment. In the groups exposed to cold and muscular exercise it reaches the normal value and in the formaldehyde group it rises above normal. After this the output falls progressively to the end of the experiment.

Fig. 2 shows the effect on chloride excretion. The total chloride output runs roughly parallel to that of water, falling to a low level at about 24-48 hr., rising again to a maximum at about 12 days and falling again progressively to the end of the experiment. However, the fall in chloride output is relatively greater than that of water. This leads to a marked decrease in chloride concentration in the first and third stages. The normal concentration is 200 mg. per 100 c.c. At the first low point the values are 82, 87 and 48 mg. in the cold, muscular exercise and

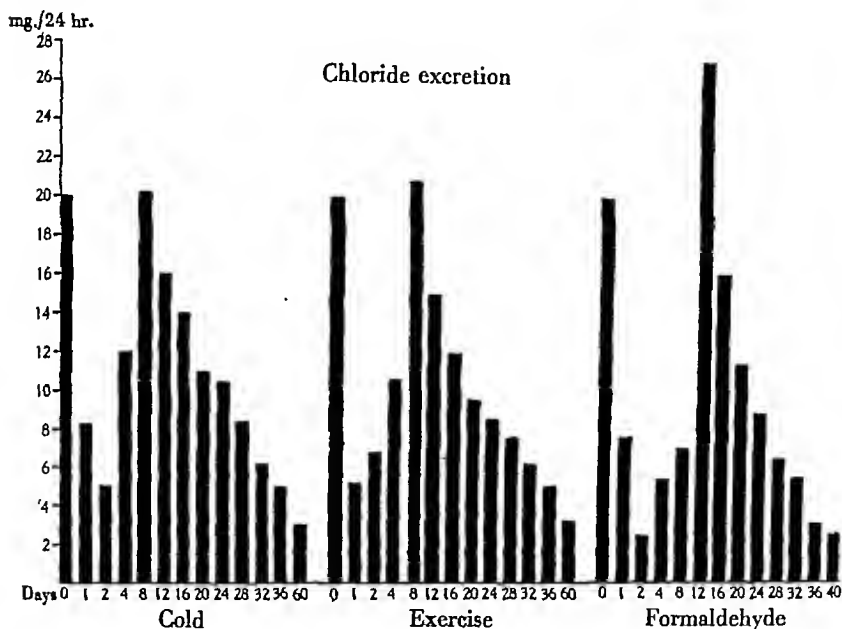


Fig. 2. The effect of exposure to cold and muscular exercise and of subcutaneous injections of formaldehyde upon urinary excretion of chloride.

formaldehyde treated groups respectively. At the maximum about the twelfth day the concentration is at or slightly above the normal level and at the end of the experiment the values in the same groups are 75, 64 and 53 mg. per 100 c.c.

The effect of cold, muscular exercise and formaldehyde on the excretion of creatine and creatinine is shown in Fig. 3. Under the influence of these stimuli the total creatinine excretion rises. This rise is due wholly to an increase in creatine excretion (shown by the black columns in the figure), while the preformed creatinine value remains unchanged or decreases slightly. The maximum point of this increase occurs 24-48 hr.

after the beginning of the exposure to the stimulus, that is during the first stage, as established by autopsy findings. The maximum appeared to be reached in 24 hr. in the group exposed to cold, in the other two groups it is reached on the second day, but the value for the second day was not determined in the case of the group exposed to cold.

The creatine excretion falls thereafter, reaching a low level 12–16 days after the beginning of the experiment. At this time it is definitely below the normal control value. The creatinine excretion also decreases from the average normal value of 5 mg. to one of about 3 mg. per 24 hr.

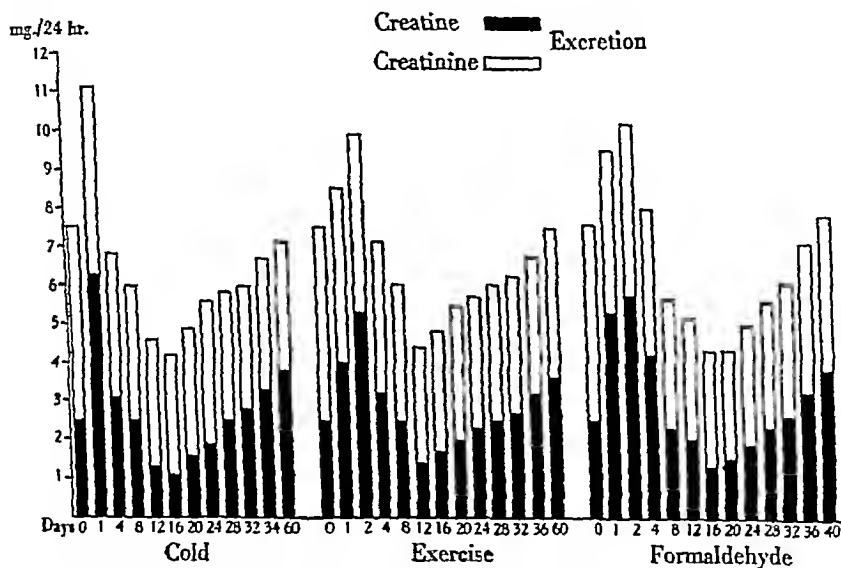


Fig. 3. The effect of exposure to cold and muscular exercise and of subcutaneous injections of formaldehyde upon urinary excretion of creatine and creatinine.

This period corresponds to the stage of "adaptation" when the morphological signs of the first stage have disappeared. After this the creatine excretion gradually rises again as the stage of "exhaustion" develops. There is a slight increase in the creatinine excretion in the groups exposed to muscular exercise and formaldehyde, but it does not reach the normal value and in the group exposed to cold the creatinine excretion does not increase during this period. The creatine excretion at the end of the experiment is slightly above normal, but does not rise as high as it does during the 24–48 hr. period.

DISCUSSION

With regard to the excretion of water Karady *et al.* [1938] stated that if, 24 hr. after an "alarm" reaction is produced by any stimulus, the same or any other stimulus be given, the second stimulus not only fails to cause further water retention but actually increases urine output. The present experiments on the other hand show no such effect of the second stimulus during the 24-48 hr. period. This difference in result may perhaps be explained by differences in the intensity of the stimulus used. For example, in the experiments of Karady *et al.* the animals were exposed to cold for 12 hr., a 12 hr. rest period was allowed to elapse and the second stimulus was then given. In those pretreated with formaldehyde 48 hr. were allowed to elapse between the first and second stimulus. In the present experiments exposure to cold was continuous and formaldehyde injections were given twice daily. The inverse response to a second stimulus reported in the previous paper has only been observed if relatively mild stimuli are used or if a rest period is allowed between the two stimuli.

Bodansky & Duff [1936] have investigated the effect of cold (3-5° C.) on the output of creatine and creatinine. They find a rise in creatine excretion. Their experiments differed from ours in that the animals received food and water throughout the experiment and no water was given by stomach tube.

The experiments reported in this communication give further support to the conception that the characteristic changes observed during adaptation are probably produced through some "common pathway", otherwise it would be very difficult to explain why entirely different agents produce similar results.

The fact that in anaphylaxis different agents may likewise elicit the same results led to similar considerations concerning the latter syndrome and later observations proved to the satisfaction of most specialists in that field that the characteristic symptoms of anaphylaxis are due to histamine liberation. The possibility that the common pathway in the general adaptation syndrome may also be due to the liberation of some tissue hormone, possibly histamine, has been considered in previous publications. Experiments to clarify this point are now under way.

SUMMARY

Experiments in the rat show characteristic changes in the excretion of water, chloride and creatine during adaptation to various noxious stimuli. These changes appear to be independent of the specific nature of

the agents to which adaptation occurs and may therefore be regarded as biochemical signs of the adaptive process as such.

The non-adapted animal, just as the animal completely exhausted by continuous exposure, responds to damaging agents such as cold, excessive muscular exercise or toxic doses of formaldehyde with a decrease in the excretion of water and chlorides and of concentration of chlorides in the urine and with a marked increase in the urinary elimination of creatine. The animal optimally adapted to the above-mentioned stimuli on the other hand shows a rise in the excretion of water and chloride towards or slightly above the normal level and a decrease of creatine and creatinine elimination below this level.

These experiments confirm the conception that during adaptation the organism responds in a characteristic manner, and that the symptoms and signs of this response are determined mainly by the intensity of the stimulus and the degree of adaptation which the organism has acquired to this stimulus.

The authors wish to acknowledge with gratitude the valuable technical assistance of Miss E. Perrault.

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THE QUICK COMPONENT OF NYSTAGMUS

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THE swift return movement of the eye in ocular nystagmus, suggesting by its briskness the sudden release of a spring, is in striking contrast with the initial slow deviation. Because of this difference, and because a certain degree of narcosis will abolish the quick twitch without affecting the slow phase, the belief has arisen that the two components of nystagmus are subserved by quite different nervous mechanisms. It is now established that the impulses responsible for the slow deviation travel from the vestibular nuclei to the nuclei of the third, fourth and sixth cranial nerves via the posterior longitudinal bundle, the vestibulo-mesencephalic tract and possibly by other pathways in the substantia reticularis [de N6, 1933; Löwenstein, 1937]. Until recently, there has been much difference of opinion as to the centres for the quick component, the suggested sites including the cerebral cortex [Bartels, 1910; Meyers, 1925], the thalamus [Wilson & Pike, 1912], the pons [Bárány, 1906], the primary vestibular nuclei [Spiegel, 1929], and the substantia reticularis [de N6, 1933]. Most authorities now agree, however, that the rapid phase arises below the level of the oculomotor nucleus and outside the vestibular nuclei. But apart from the position of the "centre" for the quick component, there is another question of interest, namely, the origin of the rhythm of normal nystagmus. Is this motor rhythm a purely labyrinthine reflex, or is it controlled also by proprioceptive discharge from the eye muscles? Certainly each quick phase appears to be a compensatory movement, restoring the eyes approximately to the position from which the slow phase had displaced them. It is reasonable to consider the possibility that the quick component is a secondary reflex, set up when afferent endings in the orbit are stimulated by the mechanical changes comprising the slow component. This explanation was first given by Bartels [1911], and is at first sight attractive. The

extrinsic ocular muscles are known to be richly supplied with sensory endings [Tozer & Sherrington, 1910; Woollard, 1931]. It may well be imagined that stretch or contraction of these muscles during the slow deviation might set up afferent impulses capable of reflexly evoking a compensatory twitch, thus producing the rapid phase. Experimental proof of this hypothesis is difficult to obtain, because it has been shown that nearly all the afferent fibres supplying the ocular muscles run in the same nerve-trunks as the motor fibres, that is, in the third, fourth and sixth cranial nerves [Tozer & Sherrington, 1910; Woollard, 1931; Tarkhan, 1933]. Tozer & Sherrington state: "the fifth nerve may send a few fibres to the muscles in the orbit, but this supply in the rabbit, cat and monkey is insignificant in amount". Hence it is impossible to cut the afferent nerve fibres supplying the ocular muscles without severing the motor fibres as well. de Kleyn [1921] tried to overcome this difficulty by paralysing the proprioceptive endings with novocaine. In a decerebrate rabbit, he cut all the nerves to the ocular muscles except the sixth nerve on one side. The movements of the innervated lateral rectus were recorded on a kymograph. Caloric stimulation of one or other labyrinth produced contractions and relaxations of the muscle corresponding to the phases of nystagmus which would have occurred had the animal been intact. Finally, de Kleyn injected novocaine into the muscle, in the hope that the sensory endings would be paralysed before the motor. He argued that if the quick component depends on impulses from the periphery, there would be a period during which the muscle would show only the slow phase, the quick twitch having been abolished by paralysis of the afferent fibres from the muscle. In his experiments no such interval occurred, however; instead, after a short period of unchanged activity, all movement in the muscle ceased. Most authorities consider that this observation disproves Bartels' theory of the "muscular" origin of the rapid phase, but, though de Kleyn's findings are most suggestive, they can hardly be regarded as conclusive. It may well be doubted that an injection of novocaine into a muscle will paralyse every afferent fibre before affecting the motor fibres; certainly there is no way of proving whether this occurs. Thus de Kleyn's conclusions rest on a supposition, which may or may not be correct. Referring to this experiment, Maxwell [1923] writes: "...it does not appear to the writer to furnish positive proof of the central origin of both sets of impulses." Another doubt concerning the possible proprioceptive origin of the quick component has been expressed by Creed [1930]. The retractor bulbi muscle, found in most lower mammals, but not in primates, consists of four slips inserted

behind the recti muscles on the equator of the eyeball. Dusser de Barenne & de Kleyn [1928] have shown that these slips can produce nystagmus after all the other eye-muscles have been cut. The retractor bulbi is supplied mainly by the third nerve, though the sixth nerve sends some fibres to the lateral slip. Creed writes: "As a result of this discovery, it may perhaps be doubted how complete was the paralysis of all proprioceptive endings in contracting muscles when de Kleyn...applied novocaine to the isolated external rectus muscle." It is clear that further evidence is required before the question can be regarded as settled. To supply that evidence has been the aim of the present investigation.

METHOD

It is obvious that to be certain of eliminating the effect of all afferent endings in the extrinsic ocular muscles, the third, fourth and sixth nerves must be cut on both sides, a procedure which necessarily interrupts the entire motor pathway to these muscles. Hence there can be no mechanical changes available for recording during nystagmus. Fortunately, valve amplification gives us a method of recording the nervous activity responsible for nystagmus, even when the ocular muscles are disconnected from the brain stem. By placing electrodes on the central stump of one of the severed nerves, one can detect the motor impulses which would have produced the changes in muscle tension corresponding to the different phases of nystagmus had their whole pathway been intact. By this means, conclusive evidence may be obtained of the effect on nystagmus of the eye-muscle afferents. Cats were used throughout this investigation. Some preliminary experiments were made to determine the feasibility of recording action-potentials in the nerves to the ocular muscles. Filaments of the sixth nerve in the orbit were found to be the most suitable fibres for the purpose. The procedure of each experiment was as follows. Under ether anaesthesia, tracheotomy was performed, and the tracheal cannula connected to an automatic anaesthetic machine which maintained indefinitely the desired depth of ether narcosis. A transverse incision was then made across the vault of the skull, extending on each side to the lateral commissure of the eyelids. The temporal muscles were detached and turned down, after which an opening was made in the skull on each side, corresponding roughly to the area of attachment of the temporal muscle. After opening the dura, the frontal and temporal lobes were gently elevated until a clear view was obtained of the third and fourth nerves, which were then cut on each side. Cutting the oculomotor nerve produced, of course, pupillary dilatation and divergent

squint. Next, the bony and cartilaginous lateral walls of the orbits were removed. The recti and obliqui muscles were defined and cut, and the four slips of the retractor bulbi muscle were isolated and divided as far back in the orbit as possible. The optic nerves and ophthalmic arteries were ligated and cut and the eyeballs removed. Each sixth nerve was then isolated at its entrance into the orbit; one was left intact, with a loop of thread round it for identification, the other was followed to its termination in the lateral rectus. One of its terminal filaments was selected for recording and secured at its distal end with a fine silk ligature, then all connexions of the nerve with the muscle were severed. The head was clamped in a steel fixation frame in such a position that the lateral semicircular canals were approximately vertical, with the ampullae uppermost (i.e. with the head extended sixty degrees from its normal posture). In this position caloric stimulation of the labyrinth produces horizontal nystagmus. The grid lead from the amplifier was connected to the nerve filament under examination. The electrode consisted of a piece of silver-chloride coated silver wire, bent at the end into a hook which passed through a loop of Ringer-soaked thread surrounding the nerve filament. In some cats, the filament was long enough to be connected to both electrodes; in others, however, the available length of nerve would only permit the attachment of the grid electrode. The earthed lead in these cases was attached to the steel fixation apparatus, so making diffuse contact with the zygomatic arches and the vault of the skull. The amplifier consisted of four battery-operated triodes, resistance-capacity coupled, leading to a power pentode with a maximum output of 7·7 W. which was able to operate simultaneously a dynamic loudspeaker and a Matthews oscillograph. The power unit was operated from the 240 V. A.C. mains by means of a transformer, a rectifying valve and suitable smoothing devices. With the amplifier at one-half its maximum sensitivity, a rectangular input potential of $10\mu\text{V}$. gave an oscillograph deflexion of 8 mm. falling to half its initial value in 0·05 sec. Most of the records were taken with the amplifier at one-half or one-quarter sensitivity. The preparation and the battery-operated stages of the amplifier were placed in a large earthed cage of fine wire-netting, to eliminate electrical interference. After listening to and recording the nerve-impulses associated with each phase of nystagmus produced by irrigation of one or other ear with hot or cold water, the intact sixth nerve was lifted up by its thread loop and cut, thus interrupting all the remaining motor fibres and almost all possible sensory pathways between the brain stem and the extrinsic ocular muscles. This procedure was carried out without disturbing the

electrodes or switching off the amplifier. The nerve discharges were again examined during labyrinthine stimulation to see if any change had occurred. Each animal was examined post-mortem to verify the completeness of the nerve section.

In a few instances, the mechanical changes in the innervated lateral rectus were recorded on the same sensitive paper as the action potentials in the opposite sixth nerve, by means of a light optical torsion myograph. In the upper record of Pl. I, Fig. 1 A, B, a contraction of the muscle produces an upstroke. In Fig. 1 A there is slow contraction followed by quick relaxation. In Fig. 1 B there is quick contraction and slower relaxation. The lower records of Fig. 1 A, B show the motor impulses in the nerve. Superimposed on the steady stream of impulses there are bursts of impulses in Fig. 1 A which are synchronous with the quick component of the nystagmus. In Fig. 1 B the muscle contracts during the quick component and the discharge of impulses in the opposite sixth nerve is synchronously diminished.

RESULTS

No attempt was made to analyse in detail the nerve action potentials, but the following observations were made, relying as much on the auditory information provided by the loudspeaker as on the oscillograph records:

(1) With the animal under light ether narcosis, there was present a tonic discharge in all the nerves examined (record A, Pl. II, Fig. 2). The action potentials were of the order of $5-15\mu V.$, and occurred at a frequency varying from 50 to 150 per sec. Since most of the nerve preparations contained a hundred or so fibres, this, of course, gives no indication of the impulse frequency in individual nerve fibres. The tonic discharge diminished or disappeared when the animal was deeply narcotized. This observation, and the disappearance of the impulses upon the death of the nerve, furnish strong evidence of their physiological origin. During the preliminary experiments, it was noticed that the tonic discharge in a sixth nerve connected to its muscle seemed to be no greater than the discharge present after severing the nerve distally. Thus it would appear that the tone of the extrinsic eye muscles, unlike that of other skeletal muscles, is very little influenced by proprioceptive impulses arising in the muscles themselves. On the other hand, labyrinthine stimulation invariably affected the tonic discharge even when narcosis was too deep for nystagmus to appear. Irrigation of the ipsilateral ear with cold water increased the frequency and intensity of the discharge; more and more groups of fibres began discharging until the noise in the loudspeaker

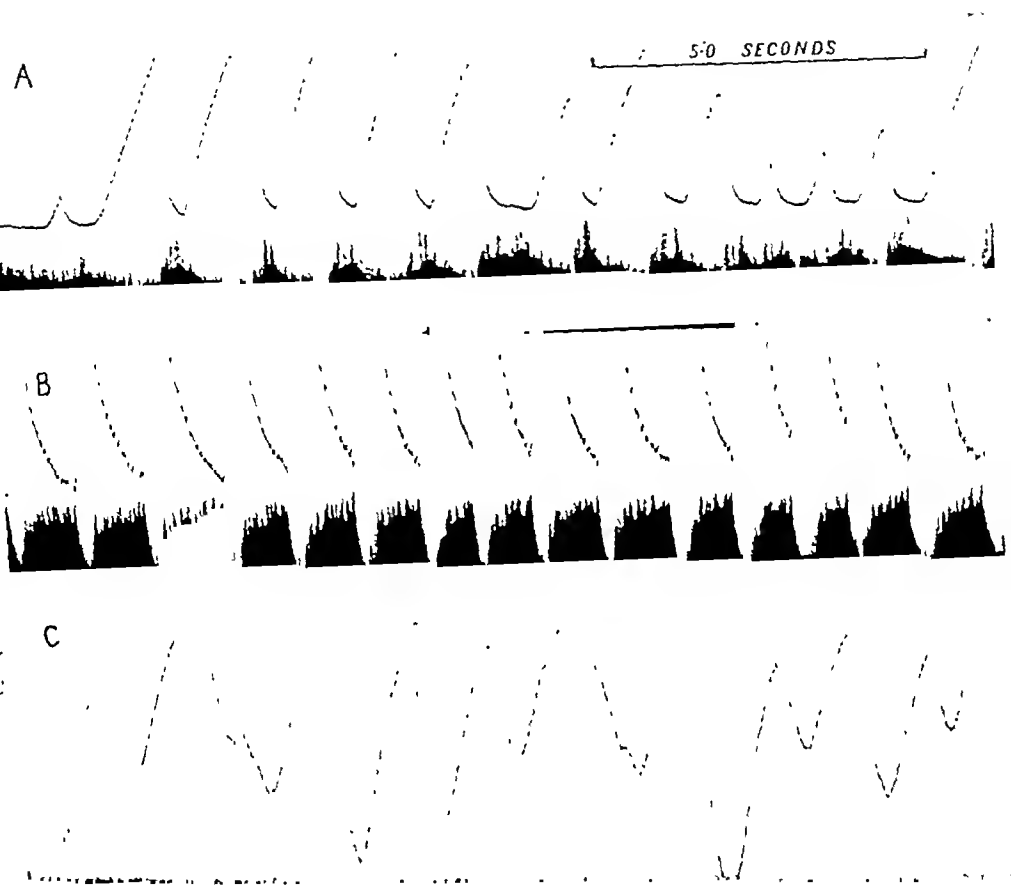


Fig. 1. Electrical activity in the left sixth nerve of a cat, photographed together with movements of the right lateral rectus muscle (the upper curve in each case). Contraction of the muscle produces an upstroke in the records, which read from left to right. A. Nystagmus with the quick component to the left. Each quick relaxation of the right lateral rectus is associated with a burst of impulse in the left sixth nerve. During each slow contraction of the muscle, the discharge of impulses gradually diminishes. B. Nystagmus with the quick component to the right. Each quick twitch of the right lateral rectus muscle is accompanied by a period of inhibition of the impulse-stream in the left sixth nerve. As the muscle relaxes, the discharge of impulses swells up to its full intensity. C. Control record of spontaneous nystagmus with the quick component to the left, taken after killing the left sixth nerve with ether. In spite of large excursions of the muscle, no associated electrical activity appears in the nerve. (Bromide paper moving at 2.3 cm./sec.)

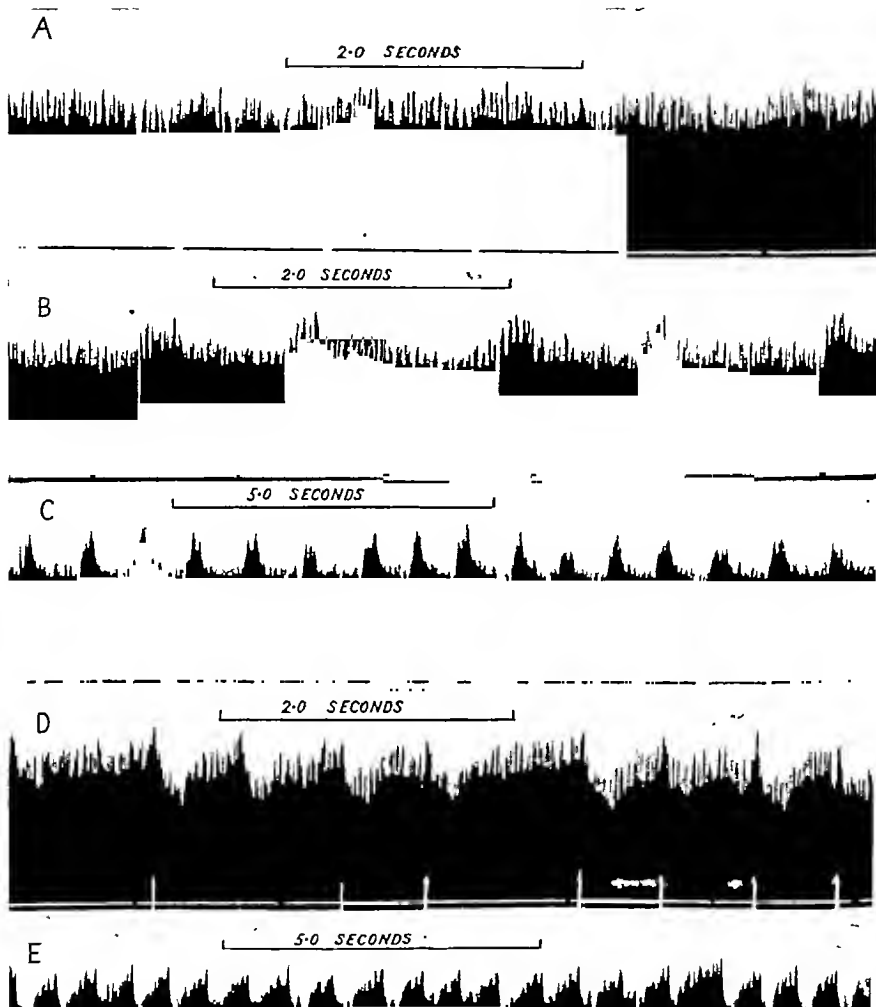


Fig. 2. Motor impulses in the right sixth nerve of a cat. A. Tonic discharge of impulses. B. "Nystagmus" to the right, showing quick component impulse-bursts (camera speed 5.2 cm./sec.). C. The same, photographed at a slower speed (2.3 cm./sec.). D. "Nystagmus" to the left, showing periods of diminished discharge corresponding to quick movements of the eyes to the left (camera speed 5.2 cm./sec.). E. The same, photographed with the bromide paper moving at 2.3 cm./sec. These patterns of electrical activity in the sixth nerve are characteristic of normal nystagmus, and undergo no change when all the nerves to the ocular muscles have been severed. All the records read from left to right.

became almost deafening and buzzing sounds indicated the presence of fibres discharging at high frequency. At this stage, irrigation of the same ear with hot water rapidly reduced the discharge to the discrete sounds of a few fibres firing at a slow rate. In some experiments, spontaneous nystagmus appeared when narcosis was very light, and often continued for several minutes. The nerve impulses associated with this spontaneous nystagmus were no different from those occurring during induced nystagmus.

(2) The impulses in the sixth nerve during nystagmus were quite characteristic. Irrigation of the right ear with hot water would normally produce a nystagmus with the slow phase to the left followed by a quick twitch to the right, that is to say, relaxation followed by rapid contraction in the right lateral rectus muscle. With the right sixth nerve connected to the amplifier, irrigation with hot water first produced a diminution in the frequency and amplitude of the tonic discharge corresponding to relaxation, followed by a loud burst of discharges corresponding to the quick twitch (Fig. 2B, C). The repetition of the cycle produced a series of bursts composed of synchronous volleys of impulses rather like those occurring in the phrenic nerve, except that each burst began abruptly and ended more gradually. At first there was only a slight tonic discharge between bursts, but as the effect of the irrigation passed off, the bursts themselves became smaller and less frequent, whilst the tonic discharge swelled up to its usual intensity.

Irrigation of the right ear with cold water would normally produce nystagmus with the quick twitch to the left, that is to say, slow contraction of the right lateral rectus followed by rapid relaxation. With the right sixth nerve connected to the amplifier, irrigation with cold water produced an increase in the amplitude and frequency of the tonic discharge representing the slow deviation of the eyes to the right. The augmented stream of impulses was then broken by a series of brief interruptions corresponding to the periods of relaxation of the muscle during the quick twitch to the left (Fig. 2D, E). This continued until the effect of the cold irrigation began to wane, whereupon the periods of inhibition became smaller and less frequent and were finally submerged in the tonic discharge. These changes in the action potentials corresponding to the quick and slow phases of nystagmus occurred as described only when the animal was lightly narcotized. With deeper anaesthesia, the bursts of impulses or periods of inhibition representing the quick component failed to appear, although there was no change in the augmentation or diminution of the tonic discharge during irrigation

corresponding to the slow phase. The slow component (tonic discharge) was abolished only by very deep narcosis.

(3) After cutting the intact sixth nerve, that is, the last sensorimotor pathway to the ocular muscles, the nystagmus action potentials were exactly the same as before. No detectable change occurred in the bursts of impulses or periods of inhibition representing the quick phases of nystagmus. In several instances, the intact sixth nerve was severed while nystagmic impulses in its fellow were being amplified, yet no alteration in the quick component impulse-bursts was ever detected during or after the procedure. Out of seven full experiments, five gave unmistakably the results just described. Labyrinthine stimulation could be repeated any number of times, always with the same effect. The other two experiments were not so clear-cut, because the results were not consistent. Although the usual variations in the tonic discharge occurred, the quick component impulse-bursts could be obtained only with the first few irrigations. However, the fact that there had been some quick component impulse-bursts after severance of all the nerves to the ocular muscles gives these two experiments some value, especially when taken in conjunction with the other results. It is possible that fatigue or damage to the brain stem was responsible in these cases for the failure of normal "nystagmus" to appear after the first few irrigations.

DISCUSSION

These observations place beyond doubt the truth of de Kleyn's assertion that the afferent endings in the ocular muscles play no essential part in normal nystagmus. In our experiments, all the efferent fibres and nearly all the afferent fibres were interrupted by cutting the third, fourth and sixth nerves on both sides. Even supposing that there were other significant sensory pathways from these muscles, they could have had no effect on the "nystagmus" in our preparations, because the necessary severance of all motor pathways prevented the occurrence of any muscle movements which might have stimulated proprioceptive endings. The retractor bulbi muscle slips were removed, and therefore need not be considered, even if some of their nerve-supply had escaped section when the third and sixth nerves were cut. It is quite clear, then, that the rhythmic phase alteration seen in normal nystagmus is entirely central in origin and could occur normally in the absence of any information from the eye muscle proprioceptors. Further, this information appears to be unnecessary for coordination of the different extrinsic eye muscles during

nystagmus. The behaviour of action potentials in the sixth nerve during nystagmus affords a striking illustration of the principle of reciprocal innervation. The gaps in the stream of impulses during nystagmus away from the side of the nerve under examination, and the fading of the tonic discharge with nystagmus of opposite direction show that reciprocal relaxation of the eye muscles does not depend on guidance from sensory endings therein.

It is interesting to note that the tonic discharge in the sixth nerve undergoes no apparent diminution when the nerve is severed distally. Thus it seems that the tonus of the eye muscles is not much influenced by the activity of their proprioceptive endings. Indeed, it would not be advantageous to the organism if the eye muscle tonus were constantly being altered by stretch reflexes, for the primary purpose of these muscles is not to keep constant the relation of the eye to the head, but to maintain as far as possible an unchanged visual field, until the cerebral cortex compels them to direct the eye elsewhere. Hence it is not surprising to find that the eye muscle tonus is determined chiefly by impulses from the ampullae of the semicircular canals and perhaps from other parts of the labyrinths [Löwenstein, 1936, 1937; Löwenstein & Sand, 1936], from the muscles, tendons and joints of the neck, and from the cerebral cortex. These impulses play on the third, fourth and sixth nerve nuclei in the brain stem.

Tozer & Sherrington have suggested that the function of the many delicate sensory endings in the extrinsic ocular muscles and tendons is to inform the cerebral cortex of the position of the eyes relative to the head. These authors have shown that one is still conscious of the position of the eyes in a dark room with the cornea and conjunctiva cocaineized on both sides. The position in the brain stem of the cells responsible for the quick phase of nystagmus remains obscure, except that it lies below the level of the third nerve nuclei and outside the primary vestibular nuclei. Lorente de N6 regards nystagmus as being a rhythmic reflex analogous to the scratch reflex. When the labyrinth is stimulated, the primary vestibular nuclei discharge a continuous series of impulses which reach not only the eye muscle nuclei, causing the slow component, but also certain neurones in the reticular substance. These neurones can discharge only intermittently, being refractory until the vestibular impulses build up by recruitment the threshold excitatory state. With each discharge of impulses from these neurones, a quick reversal of the slow phase is produced. This conception of the mechanism of nystagmus seems to offer the best explanation of the known facts.

SUMMARY

1. Action potentials in the sixth cranial nerve have been studied, by means of an amplifier and Matthews oscillograph.

2. Under light ether narcosis, a tonic discharge of impulses was present in all the nerves examined. No change could be detected in the tonic discharge after cutting the nerve distally.

3. The motor impulses in the sixth nerve accompanying both phases of horizontal nystagmus were found to be characteristic.

4. After cutting the third, fourth and sixth nerves on both sides, and extirpating the retractor bulbi muscles, labyrinthine stimulation still produced in the central stump of the sixth nerve motor impulses characteristic of normal nystagmus.

5. These experiments, therefore, show beyond doubt the truth of de Kleyn's contention that the rhythm of normal nystagmus is entirely central in origin and is independent of impulses from the ocular muscles.

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THE BLOOD FLOW THROUGH MUSCLE DURING SUSTAINED CONTRACTION

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It is generally agreed that the rate of the blood flow through contracted skeletal muscle has an important bearing on the chemical processes proceeding in that tissue and on the cause of fatigue. Nevertheless, the evidence regarding the effect of a strong prolonged contraction on the local blood flow is still conflicting. Recent experiments by Rein, Mertens & Schneider [1935], Kramer & Quensel [1937] and Bülbring and Burn [1939] show that the vessels in the strongly stimulated muscle of the anaesthetized dog are widely dilated after the first $\frac{1}{4}$ min. of contraction. This suggests that strongly contracted mammalian muscle receives a rich blood supply. There are, however, some reasons for thinking that strongly contracted human muscle does not do so. Dolgin & Lehmann [1930] found that arresting the circulation in the upper arm had no effect on the maximum length of time for which a strong hand-grip could be exerted and concluded that strong contraction arrested the flow in the muscle. Grant [1938] measured the flow in a section of the forearm plethysmographically and found that the increase during 1 min. strong hand-grip was trivial in comparison with that seen immediately after relaxation.

The object of this paper is to give a further proof of the ischaemic nature of human muscle during strong contraction.

It is self-evident that the blood flow through a single human muscle can never be measured directly by any of the existing methods. Our method shares this disadvantage, but it has the advantage that it gives qualitative results concerning the blood flow through a single group of human muscles.

PRINCIPLE OF METHOD USED TO SHOW INCREASE IN BLOOD FLOW
THROUGH THE PLANTAR FLEXORS OF THE HUMAN FOOT

Increase in blood flow through skin exposed to air at ordinary room temperature raises skin temperature. This is the basis of methods for studying the skin circulation. The underlying principle is that the exposed skin is cooler than the blood entering it; a faster flow brings more heat from the depths of the body and the skin warms up. The principle may be put in a more general way as follows. If any relatively small part of the body is above or below general body temperature, increase in its blood supply will tend to bring its temperature nearer to body temperature. The essence of the method is the existence of an initial temperature difference between the part and its blood supply.

Grützner & Heidenhain [1878] applied the principle to the study of muscle flow in anaesthetized animals. They produced the necessary temperature difference by skinning the leg; the underlying muscle cooled by exposure.

Our method is based on the same principle, adapted for showing an increase in the blood flow through the muscles of the calf of the human leg.

Muscle temperature was measured thermoelectrically, to the nearest 0.02° C., 3–6 cm. below the skin. The temperature difference between the blood and the muscle was produced by immersing the leg, up to the knee, in water. By keeping the water at a suitable steady temperature, the temperature of the resting muscle could be brought to any desired steady level, above or below body temperature.

The main difficulty was that during activity, variations in muscle temperature were caused by "heat production" as well as by blood flow. Careful controls were needed to avoid confusion. To see if a contraction increased the blood flow its effect on muscle temperature was recorded:

1. When resting muscle temperature was above blood temperature, "hot" muscle:
 - (a) With free circulation through the leg.
 - (b) While the circulation was arrested by an inflated pneumatic cuff on the thigh.
2. When resting muscle temperature was below blood temperature, "cold" muscle:
 - (a) With free circulation through the leg.
 - (b) While the circulation was arrested.

We concluded that the contraction had increased the flow through the muscle if, during its performance, muscle temperature approached the temperature of the blood entering it, that is, if it fell in 1 (a) and rose in 2 (a) and if, in addition, the temperature changes attributed to hyperaemia were absent when the contractions were made while the blood supply was arrested, 1 (b) and 2 (b).

The sustained contractions used for the study of the blood flow

Owing to the relatively simple mechanics of the movement of the ankle joint the muscles of the calf of the leg were once often experimented on to find the maximum force a human muscle could exert per sq. cm. of its physiological cross-section. To find this constant it was necessary, among other things, to determine the maximum force the gastrocnemius and soleus could exert in a voluntary effort. In general the body was loaded with weights till a point came when the heels could only just be raised from the ground. The results given by Weber [1846], Koster [1868], Hermann [1898] and Reys [1915] agree fairly well that the limiting load is about 450 kg. That is, the muscles of each calf can exert on the ball of the foot a force of about 225 kg. They calculated that the pull on the Achilles tendon was about three times as much, about 675 kg.—more than half a ton.

These data have enabled us to express the strengths of the contractions we used as fractions of the maximal strength this group of muscles can exert voluntarily. This notation will have its value for comparing our results with those obtained on other muscles.

As the gastrocnemius arises from the lower end of the femur it acts both as a flexor of the knee and a plantar flexor of the ankle. The knee joint was kept fully extended in all our sustained contractions so that the gastrocnemius exerted its pull from a standard position.

The vascular changes were examined in contractions of four different strengths 0.3, 0.2, 0.1 and 0.05 maximal.

To perform the strongest contraction, the 0.3 max., the subject stood on tiptoe, with the knee straight, on the leg in the water-bath (a dustbin). The body was just steadied with the tips of the fingers. The force on the ball of the foot was about 77 kg. (average of the body weights of the two subjects).

For the 0.2, 0.1 and 0.05 max. ones the subject sat on a bicycle saddle fixed at a convenient height beside the water-bath and, keeping his knee straight, exerted a steady pressure with the ball of his foot on a stirrup

which hung in the water near the bottom of the bath. The stirrup was attached to a suitably weighted lever. The actual forces exerted by the ball of the foot in these exercises were 45, 22.5 and 12.5 kg. respectively, according to the weight on the lever.

The 0.3 and 0.2 max. contractions, and all contractions performed while the circulation was arrested, were kept up till intolerable discomfort forced the subject to give up. This meant that muscle temperature was recorded throughout, if we may use the phrase, the whole "physiological life" of these contractions—while the muscle passed from rest to physiological (but not necessarily chemical) exhaustion.

The 0.1 and 0.05 max. contractions were given up at $\frac{1}{4}$ and $\frac{1}{2}$ hr. respectively; no acute discomfort was felt.

Rhythmic contractions

Muscle temperature changes during gentle rhythmic work were also examined. The subject transferred the weight of his body from the ball of one foot to the ball of the other once every second.

EXPERIMENTAL

Thirty-five experiments were done, each lasted on the average 5–6 hr., making a total of over 200 hr. muscle temperature observation. Protocols of typical experiments are shown in Table I.

The authors usually took turns to act as subject. Occasionally Dr N. C. Hughes—and we are very grateful to him—acted instead.

The experiment began in the morning. The dustbin, supported over a thermostatically controlled gas flame, was filled with water and the bath stirrer was started. The temperature of the water depended on whether resting muscle temperature was to be above or below body temperature. The thermojunction was sterilized by dipping it in a test tube of boiling water. The subject stood on a chair and supported most of his weight on the leg about to receive the junction. The skin of the calf was shaved and washed with surgical spirit. The observer "scrubbed up". He threaded the sterile and carefully straightened thermojunction wires into a sterile "veterinary" $1\frac{1}{2}$ in. Record mount bore 2 hypodermic needle (external diameter 1.6 mm.), and plunged the needle through the skin on the lateral or postero-lateral surface of the subject's calf deep into the muscle. In so doing he took great care to avoid the main vessels and nerves of the leg running along the posterior border of the tibia. He

TABLE I

Exercise	Subject	Date	Junc- tion depth cm.	Room temp. ° C.	Water- bath ° C.	Resting muscle temp. ° C.	Circulation free				Circulation arrested			
							Change in muscle temp. during exercise ° C.	Change in muscle temp. first after exercise ° C.	Duration of exercise min.	Resting muscle temp. ° C.	Change in muscle temp. during exercise ° C.	Change in muscle temp. during first after exercise ° C.	Change in muscle temp. during first after 3 min. ° C.	Duration of exercise min.
Rhythmic	{J.L.E.M. J.L.E.M.	2. II. 39	4	18	41.0	39.23	-1.11	+0.20	15 +	39.54	+0.43	+0.11	-2.10	2½
		23. II. 39	—	10	32.0	34.00	+2.54	0.00	15 +	34.75	+0.07	+0.03	+1.03	4
	{J.L.E.M. H.B.	23. I. 39	3	18	41.3	39.60	+0.30	-1.03	1½	39.64	+0.44	-0.03	-1.08	2
		20. II. 39	—	10	32.4	34.00	+0.17	+1.21	3	34.56	+0.28	-0.06	+1.20	4½
0-3, max. sustained contraction	{J.L.E.M. H.B.	30. I. 39	5	15	42.4	39.85	+0.29	-1.03	2½	39.82	+0.30	-0.02	-1.20	2½
		6. III. 39	3	17	32.5	34.03	+0.18	+1.01	3½	34.08	+0.16	+0.06	+1.10	3½
	{N.C.H. J.L.E.M.	17. I. 39	6	20	41.8	39.75	-0.79	-0.02	17 +	39.07	+0.53	0.00	-2.00	7
		2. II. 39	3	16	32.6	33.06	+2.21	-0.08	15 +	34.05	-0.02	0.00	+0.03	6½
0-1, do.	{H.B. H.B.	1. III. 39	4	16	42.1	40.18	-0.73	-0.01	30 +	40.17	+0.25	+0.08	-1.09	10
		24. II. 39	4	21	32.8	34.31	+2.07	-0.19	30 +	34.72	+0.15	-0.12	+0.63	15

A + sign following the duration of the exercise signifies that it was terminated for reasons other than acute discomfort.

pushed the needle home till the hilt touched the skin. He now pulled the needle out of the leg leaving the wires *in situ*. To do this successfully the wires were kept pressing into the muscle while the needle was being pulled out. When the needle was out the junction usually lay 3-6 cm. below the skin. The observer slipped the needle up the wires till it was several inches from the skin. He then bent the wires into a semicircle so that the needle could be laid flat on the skin near the puncture and fixed it to the skin with sticking plaster, so, if the wires were accidentally jerked during the experiment the strain was taken by the sticking plaster and the junction was not pulled out of the muscle. The wires were examined for kinks near the puncture, and if present, they were straightened out to ensure free movement through the skin and fascia during shortening and lengthening of the muscle.

The subject sat down on a raised chair beside the dustbin and immersed the leg with the junction in it up to the knee. The leg stayed in the water for the rest of the experiment. The observer took the temperature of the room. He recorded the muscle temperature, generally every 10 min. He checked the temperature of the water, which remained all day within a few tenths of the desired figure.

(a) In a "hot" muscle experiment, as Table I shows, the water was kept at about 42° C. Muscle temperature rose asymptotically from about 37° to about 39.5° in about 1½ hr., and then stayed almost constant, about 2.5° below the temperature of the water. It stabilized below the temperature of the water because the muscle was kept cool by the resting blood flow; arresting the circulation was soon followed by a slow steady rise in muscle temperature.

(b) In a "cold" muscle experiment the water was kept at about 32.5°. Muscle temperature stabilized very slowly, in 2-3 hr., at about 34.5°, about 2° above the temperature of the water. (Arresting the circulation stopped the warming effect of the resting flow and caused a slow fall in muscle temperature.)

As soon as the temperature of the muscle had stabilized the subject did one of the exercises described earlier. In doing the exercise the subject hardly ever felt any pain due to the wires in his leg. During the exercise, and for several minutes afterwards, the observer recorded muscle temperature at $\frac{1}{4}$, $\frac{1}{2}$ or 1 min. intervals. Then came a second long period of rest, with a light lunch, while muscle temperature settled down again. Then the circulation in the leg was arrested and, 2 min. later, the same exercise was repeated. 2 min. after the exercise the circulation was released and about an hour later the experiment finished. On some days

the experiment lasted longer, another of the exercises was done, first with the circulation free and later while it was arrested. After the experiment the length of the wires withdrawn from the leg was measured. Occasionally the insulation was cracked, if so the wires were revarnished before the next experiment (see below).

Method used for arresting the circulation. We are grateful to Dr G. W. Pickering for the specifications of the $6\frac{1}{2}$ in. pneumatio thigh cuff we used. As the exercises caused contraction of the thigh muscles which might have protected the artery, to some extent, from the pressure of the cuff, we used a cuff pressure of 230 mm. Hg; it was thrown in from a reservoir. In some control experiments the leg was made bloodless by bandaging it from the toes to above the knee with a wide rubber bandage; the cuff on the thigh was then inflated and the bandage taken off. The skin was deadly pale, and, as the strongest of our exercises had no effect on the colour, we concluded that the arrest could be relied upon to be effective in all our experiments.

The discomfort caused by the cuff was usually quite negligible compared with that which came lower in the leg towards the end of the "physiological life" of a contraction.

Thermoelectric method. Most observers have used the hypodermic needle type of thermojunction for human deep temperatures. Foged [1930] took the temperature of the venous blood with a flexible wire thermojunction which he threaded through a hypodermic needle inserted into a vein in the ante-cubital fossa. We chose the flexible wire rather than the hypodermic needle because we thought it would be less likely to break, or cause pain or injure the contracting muscle. Grant & Pearson [1933] independently adopted the same method.

In our early experiments we were sometimes troubled because the observed temperature changed very suddenly at the beginning and end of contraction. This was traced to "junction shift". During contraction the muscle thickened from side to side and the anatomical relationships of the skin and muscle changed. If the wires were gripped more firmly by the skin and fascia than by the muscle the junction was drawn a little towards the surface of the muscle. As there was a temperature gradient of about 2° C. between the muscle surrounding the junction and the surface of the leg, the movement of the junction brought it in contact with muscle at a slightly lower temperature and this caused an apparent change in muscle temperature. An equal and opposite temperature change occurred on relaxation as the junction slipped back to its original position. To fix the junction firmly in the muscle the last $\frac{1}{4}$ in. of the wires was roughened so as to catch in the muscle, and they were made as smooth as possible where they passed through the skin.

Hill [1928] used thermoelectric apparatus with a sensitivity of about 10^{-50} C. and found that if the junction was not insulated from contact with the tissue stray electrolytic E.M.F.'s caused errors. Grützner & Heidenhain [1878] stress the importance of perfect insulation, though their apparatus was only accurate to about 10^{-23} C. Our apparatus was only accurate to about 0.02° C., but we thought it easier to insulate the junction than to see whether lack of insulation caused error.

The copper-constantan thermocouple was made as follows. The constantan (Eureka) wire was 0.122 mm. in diameter and 3 m. long. Its resistance was 120 Ω , all that was necessary in the external circuit for critical damping. It was enamelled and double-cotton covered. The enamel insulated the wire from the water. Each of the two copper wires was 2 m. long, 0.274 mm. in diameter, enamelled and double-cotton covered. The cotton was stripped off all the wires for about a foot from their ends. The junction to go into the muscle was made as follows. One of the copper wires was laid alongside the constantan wire with its end level with the end of the constantan. The ends were put in a clamp fixed at the top

of a stand. A weight was hung on them about 6 in. under the clamp. This kept them parallel and in contact; they were then stuck together with several coats of Bakelite varnish. Downing [1935] has described the use of this varnish. The wires were then unclamped and the ends, which had been protected from the varnish by the clamp, were separated. The constantan was wound round the copper in a spiral of about 8 turns for $\frac{1}{4}$ in. The ends were then cut off level, hard soldered, and varnished. The spiral near the junction provided the rough surface for the muscle to grip, and the part of the wires traversing the skin and fascia was smooth and free from twists.

The constant-temperature junction was made by hard soldering the other end of the constantan wire to an end of the other copper wire, and varnishing. It was fixed near the bulb of a thermometer placed in paraffin in a vacuum flask in a gas-heated incubator kept at about 37° C. (an electrically heated incubator was tried but discarded because of stray E.M.F.'s in the thermocouple circuit). The thermometer was graduated in tenths of a degree, hundredths could be read by eye with a lens and device for avoiding parallax.

The galvanometer was a Cambridge Scientific Instrument Co. d'Arsonval. Its sensitivity (when critically damped with an external resistance of 120 Ω) was 7.6×10^{-8} V. per mm. at 1 m. The internal resistance was 8.5 Ω and period 2.4 sec. The working distance used was 1 m.

The simple circuit consisted essentially of the galvanometer, an all-copper double-pole throw-over switch, a 120 Ω copper resistance and the thermocouple. By means of the switch the galvanometer could be connected to the resistance for short-circuiting, or, to the couple for readings.

The apparatus was carefully standardized by taking several readings at known junction temperature differences. The relative accuracy of the temperatures recorded was about 0.02° C. 1° C. junction temperature difference caused a deflexion of about 50 mm. The recording time was about 3 sec., very little more than the period of the galvanometer.

Bazett & McGlone [1927] have shown that deep temperatures taken with a thermojunction are generally a little too low, as a little of the heat of the tissue round the junction is conducted away along the leads. Analogous errors may have been present in our readings. As our conclusions are based on relative and not absolute temperature changes this possible source of error is irrelevant.

RESULTS

In describing the results we shall often speak of experiments on "hot" and "cold" muscle, but it must be understood that muscle temperature was never outside physiological limits. For example, in vigorous exercise rectal temperature rises above what we shall call "hot" and it drops below "cold" in a bare leg rested for an hour or so exposed to air at ordinary room temperature.

In any given experiment we did not know for certain whether the junction was in the gastrocnemius or the soleus. J. L. E. M. judged from the direction in which he inserted the needle that the junction usually lay in H. B.'s soleus. H. B. thought he usually inserted the junction into J. L. E. M.'s gastrocnemius. Probably the temperature changes were examined many times in each muscle. Yet the changes caused by a given strength of contraction were always the same in these two subjects. We therefore think that a given strength of contraction caused similar temperature and vascular changes in both muscles.

Fig. 1, drawn from data obtained in the typical experiments shown in Table I, shows the changes in muscle temperature during the actual performance of the exercises. The top half shows the results for "hot"

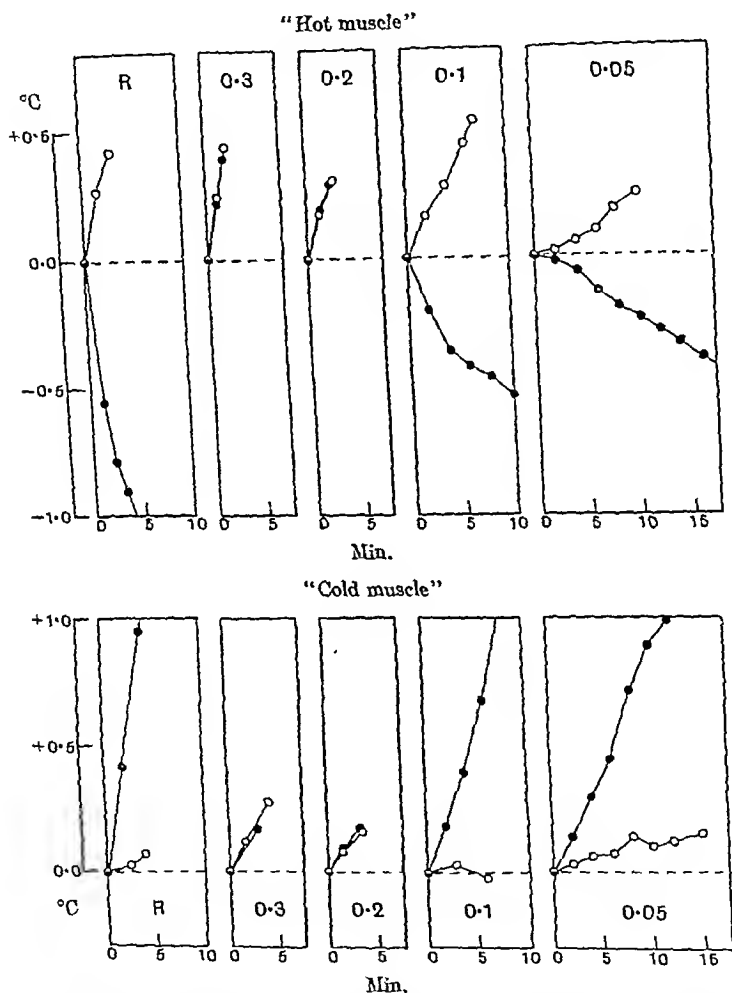


Fig. 1. Change in muscle temperature during the performance of rhythmic work, *R*, and 0.3, 0.2, 0.1 and 0.05 max. sustained contraction. • Exercise done with the circulation free. ○ Exercise done while the circulation was arrested.

muscle, resting temperature $39.5-40^{\circ}\text{C}$.; below are the results for "cold" muscle, resting temperature $34-34.5^{\circ}\text{C}$. The lines joining black dots show the temperature changes with the circulation free, those joining the circles

while it was arrested. In all cases the exercise began at time "0". The last point on each curve was taken just at the end of the exercise, when the subject said the discomfort was intolerable. With the circulation free, rhythmic work and weak static work (0.1 and 0.05 max.) could be kept up for so long that the end-point is not shown on the diagrams. Before the beginning of the exercise the temperature of the muscle had not changed by more than 0.05° C. during the previous 20 min. In any given exercise on "hot" or "cold" muscle the "circulation free" and "circulation arrested" curves are comparable, as the experiments were done on the same subject on the same day and with the junction in the same place in the calf.

Rhythmic work may be considered first. The vascular changes during this type of exercise are described here partly to illustrate how the method works, as rhythmic work is known to increase muscle flow, and partly to show the contrast between the effects of rhythmic and strong static work. Fig. 1 shows the effect of rhythmic work on "hot" muscle with the circulation free. Immediately after the work began the muscle started to cool very quickly. This could hardly have been due to an endothermic chemical change. It was more probably due to a marked hyperaemia. The relatively cold blood rushing through the hot muscle cooled it. This was confirmed by the other control experiments. When the exercise was done with "hot" muscle while the circulation was arrested the muscle did not cool, therefore the blood flow was responsible for the cooling. When the exercise was done with "cold" muscle, and the circulation free, the muscle warmed up very quickly. This is exactly what would be expected if there had been a marked hyperaemia. The muscle would be warmed by the rush of relatively warm blood. This is checked by the fact that the rapid rise in temperature did not occur when the exercise was done on "cold" muscle while the circulation was arrested.

Taking this evidence as a whole it amounts to proof that the rhythmic work was accompanied by increase in muscle flow, as would be expected on general grounds. Barcroft & Kato [1915] have shown that short repetitive bursts of tetanic stimulation increased the flow through the dog's gastrocnemius.

Some further points may be added. The curves show that while the circulation was arrested the work could not be kept up for as long as 5 min. With the circulation free it was kept up for 15 min. quite easily and stopped before any distress was felt. Evidently the circulation plays a very important functional role in the performance of rhythmic work, as has already been shown by Lewis, Pickering & Rothschild [1931]. As

will be seen later the circulation through muscle is of no functional importance in strong static work.

The rise in muscle temperature during exercise with the circulation arrested is interesting though it is unnecessary, for the present purpose, to discuss its cause in detail, and we merely give the probable explanation. The principal factors concerned are (a) "heat production" due to the rapid metabolism accompanying the activity, (b) the removal, by the arrest, of the thermal influence of the resting blood flow. Arresting the blood flow through "hot" muscle removes the cooling effect of the resting flow so the muscle temperature gradually rises towards that of the water in the bath. Arresting the circulation in "cold" muscle stops a warm resting flow and the muscle begins to cool towards the water temperature. That is, in a "hot" muscle experiment the rise in temperature during exercise with the circulation arrested is due to "heat production" + heat entering the limb from the water. In a "cold" muscle experiment the rise in temperature is due to "heat production" - heat loss from the limb to the water. In a "hot" muscle experiment the "heat production" is exaggerated, in a "cold" one it is reduced. The same applies to the temperature changes in the other exercises performed during arrest, and it will not be necessary to draw any further attention to this point.

We may now turn to the results obtained with the 0.3 and 0.2 max. sustained contractions, results which are entirely different from those of rhythmic work. The two points shown in Fig. 1 are: (a) during the exercise muscle temperature rose at practically the same rate in each "hot" or "cold" experiment whether the circulation was free or arrested—there was no evidence of hyperaemia. If the flow had stopped altogether during contraction the results would presumably have been those seen in Fig. 1; we cannot be sure that the flow was brought to an absolute standstill, but it is certain that it did not rise much above the resting rate, which, in the experiments on cold muscle, must have been extremely small, say 1 c.c./100 c.c. muscle [Grant & Pearson, 1938]. (b) All these exercises were kept up till the subject could bear them no longer. The "physiological life" of these contractions was the same whether the femoral vessels were patent or occluded, the blood flow through the muscle, if any, was of no functional significance.

On the other hand, Fig. 1 shows that the results for the weaker 0.1 and 0.05 max. contractions resembled those of rhythmic work. With the circulation free a very distinct hyperaemia occurred during the exercises; the "physiological life" of these contractions was shortened by arrest, the hyperaemia had a functional value.

To sum up, 0.3 and 0.2 max. contractions of the flexors of the foot are not accompanied by hyperaemia, the flow is probably arrested in the muscle; 0.1 and 0.05 max. contractions and rhythmic work are accompanied by marked hyperaemia.

The vascular changes during the recovery period may be inferred from the muscle temperature changes during the first 3 min. after exercise, given in Table I. Very marked hyperaemia set in a few seconds after the 0.3 and 0.2 max. contractions relaxed. On the other hand, the hyperaemia accompanying the 0.1 and 0.05 max. contractions and the rhythmic work did not become accentuated after the exercise.

Table I shows the temperature changes during the first 3 min. following release of the circulation. Marked reactive hyperaemia always occurred.

DISCUSSION

The experiments just described establish the fact that a single group of human muscles, the plantar flexors of the foot, are almost or quite ischaemic during strong contraction (0.3 and 0.2 max.) and are markedly hyperaemic during weak contraction (0.1 and 0.05 max.). The facts are probably explained as follows. Anrep & v. Saalfeld [1935] and Grant [1938] have recently added to the evidence in favour of the liberation of vaso-dilator substances in active muscle. We attribute the hyperaemia during weak contraction mainly to the action of these metabolites. There seems to be only one possible explanation for the suppression of the hyperaemia during strong contraction, namely, the compression of the potentially dilated vessels by the taut muscle fibres, and this is borne out by the immediate appearance of the hyperaemia a few seconds after the muscle relaxes. Conclusive evidence already exists to show that contracted muscle can impede the blood flow mechanically, both in man and in the dog [Lindhard, 1920 *a, b*; Anrep, 1935; Kramer & Quensel, 1937; Grant, 1938]. The critical strength of contraction of the plantar flexors of the foot above which the hyperaemia was latent till relaxation was about 0.15 max., equivalent to the exertion of a force of about 35 kg. on the ball of the foot, with a corresponding strain of about 100 kg. on the Achilles tendon, conditions which would be fulfilled in an adult standing on tiptoe on both feet.

Our results will now be discussed in relation to other work. Dolgin & Lehmann [1930] found that weak hand-grips could be exerted for longer when the circulation in the arm was free than when it was arrested, but that the performance of strong grips was unaffected by the arrest. They concluded that the circulation in the active muscle was maintained

and was of functional value during the weak grips, but that during the strong grips the muscles became ischaemic. Our experiments support their conclusions. Asmussen & Hansen [1938] recently extended earlier work by Lindhard [1920 *a, b*] and found that the rate of pulmonary oxygen intake increased both during and after sustained contractions so strong that they could only be exerted for 2 or 3 min. They concluded that the flow through the active muscles increased both during contraction and again after relaxation when the mechanical opposition to the flow had vanished. At first sight these results seem to conflict with the ischaemic nature of strong contraction indicated by our experiments. It seems likely, however, that in Asmussen & Hansen's experiments the pulmonary oxygen intake would be the composite expression of events proceeding in a very large number of muscles in the legs and adjacent parts of the trunk and that the strength of contraction would differ considerably from one muscle to another. If that were the case the most powerfully contracted muscles might be ischaemic and the increased oxygen intake might be associated with the greatly increased flow through those muscles which were not contracting so vigorously. Similarly, the slight increase in blood flow found by Grant [1938] in a section of the forearm during the exertion of a strong hand-grip may have been the expression of a marked hyperaemia in some muscles and an ischaemia in others.

Rein *et al.* [1935], Kramer & Quensel [1937] and Bülbring & Burn [1939] found that during prolonged strong nervous stimulation of the anaesthetized dog's gastrocnemius muscle the vessels became widely dilated. In the plantar flexors of the human foot this finding would be expected during weak but not during strong contraction. Two alternative explanations seem possible. There may be a genuine difference in the behaviour of the muscle flow during strong contraction in human and in dog's muscle. This seems rather unlikely. Alternatively the contractions studied in the animal experiments may actually have been weak and not strong ones. In the dog experiments a contraction elicited by maximal nervous stimulation has been assumed to be a strong one. The assumption is a natural one, but does not seem entirely self-evident in the light of a critical examination of the actual pull exerted by the maximally stimulated muscle. As far as we are aware the maximum motor tetanus of the decerebrate dog's gastrocnemius is unknown. Eccles & Sherrington [1930] found an average value of 12.5 kg. for cats averaging 3.5 kg. in weight, which suggests a value of approximately 30–40 kg. for 10 kg. dogs. Kramer & Quensel [1937] do not mention the strength of the

contractions studied in their experiments. Rein *et al.* [1935], if we interpret their experiments correctly, tied the Achilles tendon to a 1 kg. weight, so that the tension developed during contraction could not have exceeded that amount. Bülbring & Burn [1939] recorded isometric tensions of about 18.5 kg., but these soon fell to a steady level of about 5 kg. Anrep & v. Saalfeld [1935] recorded, on the same muscle, an isometric tension of 2.7 kg. during a contraction lasting 5 sec. These figures suggest that the contractions elicited in the above experiments on the anaesthetized dog may not have been strong ones. The idea gains probability for another reason. The highest recorded steady tension in the above experiments was 5 kg. As the force exerted on the pad of the dog's foot is about one-third of that applied to the Achilles tendon, 5 kg. on the tendon would correspond to 1.7 kg. on the pad, this would not be sufficient to support one-quarter the weight of a 10 kg. dog. It seems possible that some factor common to all the experiments on the anaesthetized dog may have prevented strong stimulation from eliciting strong contraction; if it elicited weak contraction the accompanying hyperaemia would correspond to that also found during weak contraction of the flexors of the human foot.

SUMMARY

1. Recent work shows that the needs of strongly contracting muscle are met by a poor vascular response in man but a liberal one in anaesthetized animals.

2. The effect of sustained contraction on the blood flow through a single group of human muscles, the plantar flexors of the foot, has been examined qualitatively by a new method.

3. 0.05 and 0.1 maximal contractions of this group are accompanied by marked hyperaemia which gradually subsides during the recovery period.

4. The length of time for which contractions of this strength can be maintained is very much shortened by previous arrest of the circulation in the leg, therefore the hyperaemia normally accompanying them is of functional value.

5. 0.2 and 0.3 maximal contractions are not accompanied by hyperaemia; the flow is probably arrested in the muscle. Marked hyperaemia sets in a few seconds after relaxation.

6. The length of time for which contractions of this strength can be maintained is unaffected by previous arrest of the circulation, therefore the blood flow through the active muscle, if any, is of negligible functional value.

7. The suppression of the hyperaemia during the stronger contractions is believed to be due to compression of the potentially dilated vessels between the taut muscle fibres, this becomes a dominant factor when the strain on the Achilles tendon exceeds about 100 kg.

8. These conclusions are believed to be consistent with the previous results obtained on man. The reason for the discrepancy between the experiments on human and animal muscle is discussed.

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GASTRIC CARBONIC ANHYDRASE

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IN their study of the secretion of hydrochloric acid Davenport & Fisher [1938] discovered large amounts of carbonic anhydrase in the gastric mucosa of cats, rats and rabbits. In order to find whether or not the enzyme has anything to do with the secretion of acid it is necessary to determine in what cells the enzyme is present.

It is generally believed that the parietal cells secrete the acid or its precursor. Bernard [1859], Harvey & Bensley [1912], Fitzgerald [1910] and Collip [1920] injected ferric salts and ferrocyanide intravenously in the hope that Prussian blue would be precipitated at the site of the formation of the acid. Prussian blue always appeared on the surface of the gastric mucosa, and it was occasionally found in the lumen of the glands and in the canaliculi of the parietal cells. Harvey and Bensley believed that the Prussian blue was precipitated only in moribund cells and that its presence in only a few cells was not proof that acid is secreted by all the parietal cells. Fitzgerald & Collip accepted the presence of Prussian blue as proof that the parietal cells do secrete acid.

Harvey & Bensley, Dawson & Ivy [1926] and Hoerr & Bensley [1936] stained with neutral red or cyanamin pieces of mucosa clipped from actively secreting stomachs and examined the cells microscopically. Chambers [1915] injected neutral red directly into the parietal cells with a micropipette. Dawson & Ivy claimed that if the cells be observed within 2 min. after excision a transitory acid colour can be seen. The other workers claimed that the cells and the secretion in their canaliculi were always neutral if not alkaline. These experiments are inconclusive, because the acid, if present, would be neutralized by diffusion during the interval

between excision and examination. In fact, the observations depend upon the diffusion of the dye into the cells and canaliculi, and one would expect that the acid might equally well diffuse out at the same time.

Glick, Holter, Linderstrom-Lang & Soeborg [1935] found that acid of undetermined nature has a distribution in the gastric mucosa roughly similar to that of the parietal cells. They also found that the distribution of chloride is very similar to that of the parietal cells. Fitzgerald found chloride abundant in the parietal cells, and Collip found chloride present during activity though absent when the cells were inactive. Gersh [1938] claimed that chloride is absent from the cytoplasm of all gastric gland cells. Glick *et al.* [1935] remark that "on the basis of the available material it is only possible to assert that the parietal cells are in all probability the cells which produce hydrochloric acid".

METHODS

Cats were killed by bleeding after ether anaesthesia and rats by a blow on the head. A canula was at once inserted into the abdominal aorta, and the visceral blood vessels were perfused with 0.9% saline containing 6% gum arabic. The addition of the gum arabic prevented or reduced the oedema occurring during perfusion. When the blood vessels were seen to be free of blood the stomach was opened and the mucosa examined. The mucosa was usually found to be clear yellow in colour, and any sample suspected of not being free of blood was discarded. Aqueous extracts were made of the mucosa and tested for enzyme activity.

Carbonic anhydrase was determined according to the method of Meldrum & Roughton [1933]. The apparatus was used at 0° C. and at atmospheric pressure, and it gave results consistent and reproducible to within $\pm 5\%$. The activity was calculated and expressed as enzyme units (*E*) according to the method of Meldrum & Roughton, but no correction was applied to bring the calculated activity to 15° C. The enzyme unit used in this work is therefore two to three times smaller than that of Meldrum & Roughton.

The activity of the extracts was caused by the presence of carbonic anhydrase since they catalysed the hydration and dehydration of carbon dioxide. The addition of the extracts to the phosphate buffer used in the rate measurements did not change the *pH* of the buffer as measured by a glass electrode. The end-points of the catalysed and uncatalysed reactions were the same. The activity of the extracts was completely destroyed by

30 sec. boiling, by 20 min. at 60° C., and by 30 min. at pH 2 or 13. The activity was inhibited by $M/800$ HCN and by the specific inhibitor of Booth [1938].

The activity of the extracts was not caused by included blood. The active extracts gave a negative benzidine test. Extracts to which very much less blood had been added than would be needed to account for the activity gave strongly positive tests. Extracts from the various parts of the mucosa showed a characteristic distribution of activity, and it is unlikely that the distribution of included blood would be always the same. Some slices were found to contain 3–5 E per c.mm. Had the activity of the slices been caused by included blood the amount of blood would have been equal to the volume of the slices. Upon histological examination of over a hundred slices of perfused mucosa small clumps of red blood cells were seen on only three occasions.

Distribution of carbonic anhydrase

Cylinders were stamped from the gastric mucosa by means of a cork borer. Extracts were made of the cylinders, and their carbonic anhydrase

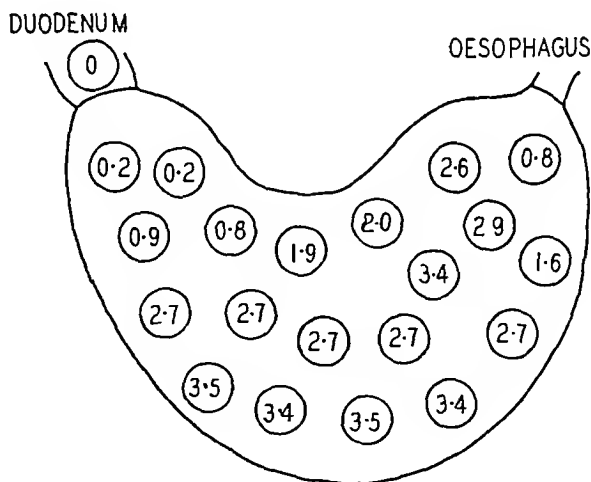


Fig. 1. Cat stomach. The figures in the circles represent the average carbonic anhydrase concentration in E per mg. wet weight.

activity was measured. In Fig. 1 the distribution of the enzyme in a cat's gastric mucosa is shown, and in Fig. 2 the distribution in a rat's gastric

mucosa. In both animals the distribution is roughly similar to that of the parietal cells which are abundant in the fundus, more rare in the cardia and completely absent from the pyloric antrum.

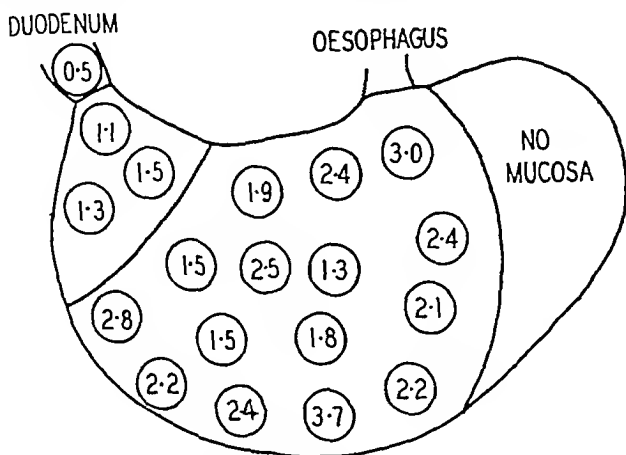


Fig. 2. Rat stomach. The figures in the circles represent the average carbonic anhydrase concentration in E per mg. wet weight.

The cell-counting method

A modification of the method of Glick *et al.* [1935] was used to determine in what cells the enzyme is present. Cylinders 4.00 mm. in diameter were stamped from various parts of the mucosa and placed on the table of a freezing microtome in such a position that the plane of cutting was perpendicular to the axis of the cylinder and to the lumina of the glands. The tissue was frozen, and slices 0.020 mm. in thickness were cut. Cutting was continued until *three consecutive sections* were obtained which were satisfactory. All possible precautions were taken to ensure uniform thickness of the slices. The groups of three sections were distributed almost at random with respect to distance from the surface of the mucosa.

The 1st and 3rd sections were ground in a mortar made of a small test tube. 3 c.c. of the $M/5$ phosphate buffer, pH 6.8, used in the rate measurements was added, and the slices were thoroughly extracted. 2 c.c. of the extract were then added to one compartment of the boat of the carbonic anhydrase apparatus, 2 c.c. of $M/5$ NaHCO_3 to the other compartment, and the carbonic anhydrase concentration was determined.

The 2nd section was mounted on a microscope slide, fixed in Carnoy's solution and stained with Delafield's haematoxylin and aurantia. A camera lucida outline drawing of the section was made, and careful note was taken of its folds and other imperfections. The area of the drawing

was measured with a simple planimeter, and from the known magnification the area of the slice on the slide was calculated. In sixty-four slices the measured area had a mean of 12.9 sq. mm. The area calculated for a circle 4.0 mm. in diameter is 12.6 sq. mm. There was considerable variation in the area of the slices which depended on the amount of stretching occurring when the slices were straightened on the slide.

The slice was observed under an ocular of $5\times$ and an oil immersion objective. The total area of such a visual field was found to be 0.0407 sq. mm. By means of a mechanical stage the slice was moved in a systematic manner from left to right and from top to bottom, so that fields could be observed over the whole slice. There was no selection of the fields, and the method of moving the stage provided that the fields observed were a fair sample of the whole slice. From twenty to forty fields were observed on each slice.

In each field the total number of cells of one type was counted and recorded. The parietal cells were recognized as large, clear yellow cells with more or less central nuclei, and they were situated at the periphery of the glands. The chief cells were smaller, brown in colour with a deeply stained reticulum.

The total number of cells counted in all the fields was multiplied by the total area of the slice and divided by the total area of the fields counted. The result was taken to be a good estimate of the number of cells of one type in the slice. This method eliminated the effect of the variation in the size of the slices. With considerable stretching the slice would be bigger, but the density of the cells in the fields counted would be correspondingly less. The reverse would be true where no stretching took place. There was no systematic relation found between the size of the slice on the slide and the cell count or enzyme concentration.

The number of cells of one type in a slice was plotted against the observed concentration of carbonic anhydrase in the two surrounding slices. The regression line for the data was calculated, and the correlation coefficient was calculated. Since the correlation coefficient is a statistic whose distribution departs from the normal distribution it is useless to calculate its standard error [Fisher, 1936]. However, the correlation coefficient can be transformed into the statistic z whose distribution is approximately normal and whose standard error is $\sqrt{1/(n' - 3)}$, where n' is the number of pairs of observations. There is only one chance in a hundred that the value of z lies outside the calculated value ± 2.977 times its standard error. Therefore the limits of z can be calculated and from them the limits of the correlation coefficient.

There are two fundamental assumptions involved in this method. The first is that the concentrations of enzyme in the cells of any one type are distributed at random about a mean. It is not possible to test this assumption, but it seems entirely reasonable. The second assumption is that the number of cells in the second section is equal to the average number of cells in the 1st and 3rd slices, thereby providing an accurate estimate of the number of cells in those slices. This assumption was tested by counting the parietal cells in several sets of serial sections made and counted as described above. The results are given in Table I. It can be seen that the assumption is justified.

TABLE I. Parietal cell counts on serial sections

No. of slice	Cells found	Mean of surrounding slices	% difference
1	6510		
2	5750	5680	+ 1.2
3	4850		
1	6450		
2	6030	6065	+ 0.2
3	5680	5685	+ 0.1
4	5340		
1	10350		
2	11000	11950	+ 8.6
3	13550	12200	- 10.0
4	13400	12525	- 6.5
5	11500		
			<hr/> - 1.1

RESULTS

The first data were derived from the gastric mucosa of the cat. It was found that the slices fell into two distinct groups. The first group was composed of slices cut from the base of the glands, and they contained only parietal cells, chief cells and connective tissue. The second group contained slices cut from nearer the surface, and in addition to parietal cells and chief cells they contained gastric pits made up of long tapering surface cells. The two groups will be considered separately.

For the first group the parietal cell count is plotted against the enzyme concentration in Fig. 3. The line drawn is the regression line, and its equation is

$$Y = 0.000102x - 0.036,$$

where Y is the enzyme concentration in E per slice and x is the number of parietal cells per slice. The correlation coefficient is $+0.95$, and there is less than one chance in a hundred that its true value lies outside the limits of $+0.81$ to $+0.99$. The correlation is extremely high.

It was not possible to count other types of cells, e.g. Heidenhain's cells

or the argentaffine cells. The chief cells comprised 95-99% of the total number of cells in these slices, and consequently small errors in counting the chief cells would render any conclusions based on their counts meaningless. It is unlikely that any other type of cell would have perfect correlation with the parietal cells over the whole range of from no cells to 10,000 cells per slice. This would be necessary if the enzyme really were in another type of cell, and the correlation between enzyme and parietal

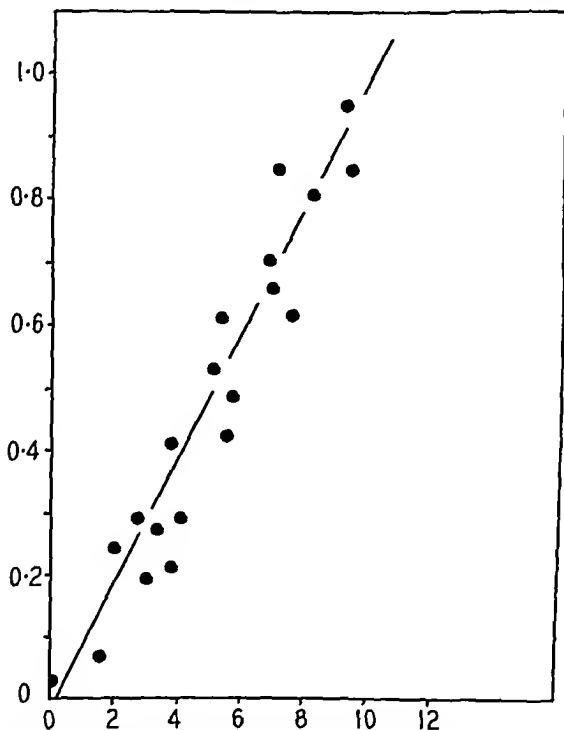


Fig. 3. Abscissae: 1000 parietal cells per slice of cat gastric mucosa. Ordinate: carbonic anhydrase in *E* per slice.

cells were only apparent. The intercept on the *Y*-axis is negligibly different from zero, so it is improbable that in these slices there is any carbonic anhydrase in any other type of cell. Consequently it can be concluded that carbonic anhydrase is confined to the parietal cells.

Cat blood contains about 3.7 *E* per c.mm. From the equation above it is seen that one million parietal cells contain 102 *E*. The average diameter of the cells is 0.017 mm., and assuming them to be spherical the volume of one million cells is 2.6 c.mm. The parietal cells contain about

39 E per c.mm. or about five to six times as much as an equal volume of red blood cells.

In the group containing surface cells the enzyme concentration was obviously not proportional to the number of parietal cells. This led to the supposition that some carbonic anhydrase might be in the surface cells. The parietal cells were counted, and from the equation above the most probable amount of enzyme in that number of parietal cells was calculated.

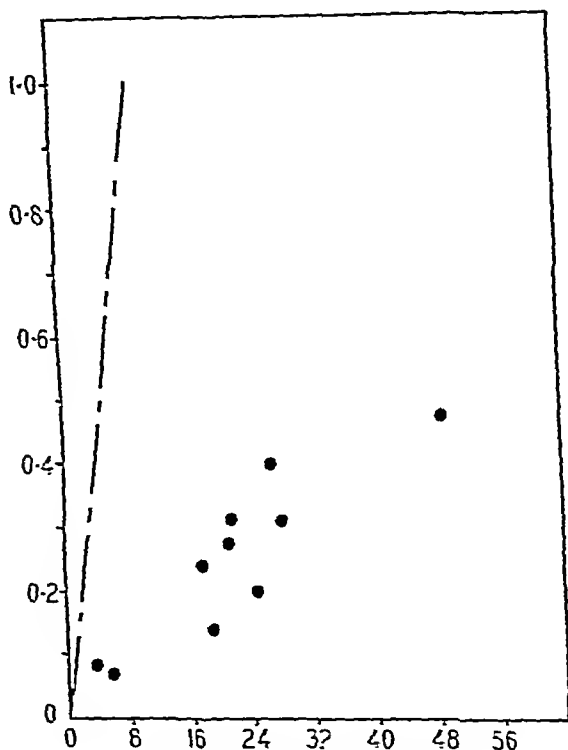


FIG. 4. Abscissae: 1000 surface cells per slice of cat gastric mucosa. Ordinate: residual carbonic anhydrase in E per slice.

This was subtracted from the total amount of enzyme. The surface cells were counted, and their number per slice is plotted in Fig. 4. The broken line is the parietal cell line plotted on the same scale. It can be concluded that there is about a tenth as much enzyme in the surface cells as in the parietal cells. This conclusion is supported by several observations that slices containing only surface cells contained about 0.4 E per slice.

There was no difference between pairs of observations made on fasting cats and on cats digesting a large meal. This indicated that if the enzyme

is secreted it is in amounts small in comparison with the amount in the cells. A search for carbonic anhydrase in the gastric juice revealed only very small amounts which may have come from traumatic blood or desquamated surface cells.

When slices were made from rat gastric mucosa none containing surface cells was found. The surface epithelium is so thin that it was cut off before satisfactory slices were obtained. When slices were cut obliquely

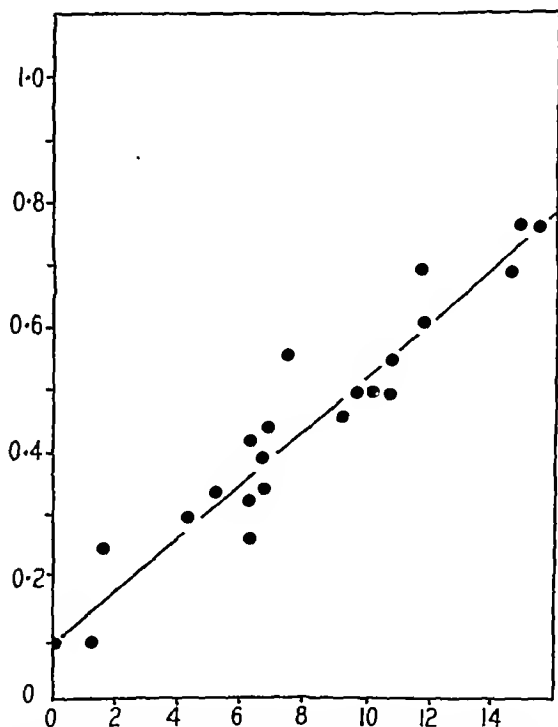


Fig. 5. Abscissae: 1000 parietal cells per slice of rat gastric mucosa. Ordinate: carbonic anhydrase in *E* per slice.

the surface cells fell off before the slices could be mounted and fixed. Consequently the only data are derived from slices containing chief cells and parietal cells.

In Fig. 5 the carbonic anhydrase in *E* per slice is plotted against the parietal cell count per slice. The equation of the regression line is

$$Y = 0.000043x + 0.10.$$

The correlation coefficient is +0.95, and the limits are +0.81 to +0.99. Again the correlation is extremely high.

There was no correlation found between the count of the chief cells and the enzyme concentration.

Rat blood contains about 1.7 *E* per c.mm. One million rat parietal cells contain 43 *E* or about 17 *E* per c.mm., which is about three times as much as an equal volume of rat red blood cells.

There was no difference between pairs of observations made on fasting and on digesting rats. Minute amounts of carbonic anhydrase were found in the gastric juice of rats, and the enzyme might have come from traumatic blood or desquamated cells.

It is seen from Fig. 2 that there is a small amount of enzyme in the pyloric antrum of the rat. There are no parietal cells in the pyloric antrum. The intercept on the *Y*-axis of the regression line for the rat parietal cells is significantly different from zero which means that there is a small amount of carbonic anhydrase not included in the parietal cells and more or less uniformly distributed throughout the mucosa. The amount is about 0.4 *E* per mg. wet weight. This amount of enzyme together with a small amount in the surface cells of the pyloric antrum would account for that found in the pyloric antrum. It was not possible to obtain satisfactory slices of the thin and friable mucosa of the antrum, so this supposition could not be tested by the cell-counting technique.

That there is some carbonic anhydrase in the surface cells of the rat fundus is indicated by the following observations. The relation between enzyme concentration and distance from the surface was found by the analysis of serial sections. This was compared with the relation between the parietal cell count and the distance from the surface as found from counting serial sections. The latter relation was confirmed by observations made on paraffin sections cut in planes perpendicular to the surface. The distribution of parietal cells was found to be the same as that found by Glick *et al.* for the pig's stomach. The two relations were found to be similar for distances corresponding to the necks of the glands or deeper, but at a distance corresponding to the depth of the gastric pits there was considerable carbonic anhydrase but no parietal cells.

Similar observations made on the mucosa of the pyloric antrum showed that there was carbonic anhydrase in the surface cells and in some unidentified cells deeper in the glands.

DISCUSSION

Evidence that there is a relation between blood carbon dioxide and gastric secretion is present in the work of Delhougne [1927] who found decreased acidity in response to a test meal after hyperventilation and in

that of Bakaltshuk [1928] who found increased gastric acidity after inhalation of carbon dioxide. Apperly & Crabtree [1931] found that the total acid secreted varies with the blood carbon dioxide. Brown & Vineberg [1932] found that decreases in blood carbon dioxide inhibit acid secretion following vagal stimulation, and Dodds & McIntosh [1923] found that blood carbon dioxide rises during gastric secretion.

The fact that there is a high concentration of carbonic anhydrase in the parietal cells leads to the supposition that carbon dioxide has an important role in the mechanism of the formation of the acid. It is unlikely that blood carbon dioxide is used directly by the parietal cells, for this would require either that the concentration of carbon dioxide in the parietal cells be lower than that in blood or that a mechanism be present in the cells for concentrating it against a diffusion gradient. It is more likely that carbon dioxide is produced within the cells from the combustion of a metabolite, and the higher the blood carbon dioxide the higher the concentration that can be reached in the cells. Carbon dioxide arising in the cells would be immediately hydrated to carbonic acid. The subsequent ionization of the carbonic acid might provide the hydrogen ions for the hydrochloric acid.

The apparent first ionization constant of carbonic acid is given by the equation

$$K_{\text{CO}_2} (\text{dissolved } [\text{CO}_2] [\text{H}_2\text{CO}_3]) = [\text{H}^+] [\text{HCO}_3^-].$$

The accepted value of the constant is 3×10^{-7} . Dissolved $[\text{CO}_2]$ at equilibrium is of the order of 1000 times greater than the $[\text{H}_2\text{CO}_3]$. Consequently the true first ionization constant of H_2CO_3 as defined by the equation

$$K_{\text{CO}_2} [\text{H}_2\text{CO}_3] = [\text{H}^+] [\text{HCO}_3^-]$$

is larger and is about 2×10^{-4} [Thiel & Strohecker, 1914]. Hydrochloric acid is secreted at a concentration of $0.17M$ [Hollander, 1934]. The concentration of bicarbonate in the cells is unknown, but it is unlikely that it is much greater than that in gastric venous blood which is about $0.020M$ [Hanke, Johannesen & Hanke, 1931]. Substituting these two values in the equation for the true first ionization of carbonic acid it is found that $[\text{H}_2\text{CO}_3]$ is required to be 17 if $0.17M$ hydrochloric acid is to be formed by mass action as has been supposed by Maly [1878] and others. The quantity $[\text{H}_2\text{CO}_3]$, of course, represents the activity of carbonic acid, and we know nothing of the activity of carbonic acid in the parietal cells. However, 17 is an inconceivably high value. Another mechanism must be found which is capable of concentrating acid against a diffusion gradient and of providing the necessary energy. A more detailed and critical review of this problem is in preparation.

SUMMARY

Carbonic anhydrase is present in the parietal cells of the gastric mucosa of cats and rats. In cats the concentration of carbonic anhydrase in the parietal cells is five to six times higher than in the red blood cells; in rats it is about three times higher.

Carbonic anhydrase is present in the cells of the surface epithelium of the gastric mucosa. In cats its concentration in the surface cells is about a tenth of that in the parietal cells.

There is no carbonic anhydrase in the chief cells of cats or rats, and if any carbonic anhydrase is present in any other type of cell in the fundus it is in very low concentration.

A small amount of carbonic anhydrase is present in an unidentified type of cell in the pyloric antrum of the rat.

I wish to thank Dr Henry Borsook for his encouragement and advice and Dr R. B. Fisher for his many suggestions and help with the statistical analysis of the data. I am also greatly indebted to Dr F. J. W. Roughton for allowing me to use and to copy his apparatus and to Dr V. H. Booth for instructions as to its use.

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RESPONSES WHICH NERVE IMPULSES EVOKE
IN MAMMALIAN STRIATED MUSCLESBY J. C. ECCLES AND W. J. O'CONNOR¹*From the Kanematsu Memorial Institute of Pathology,
Sydney Hospital, Sydney**(Received 9 June 1939)*

MUCH experimental evidence has now been accumulated in support of the hypothesis that neuro-muscular transmission in striated muscle is brought about by acetylcholine which nerve impulses cause to be secreted at the region of the motor end-plate. However, a proper critical evaluation of this evidence has hitherto been impracticable on account of our ignorance of the physiological events which are concerned in neuro-muscular transmission. For example, Brown's observations [1937] on the action of eserine on neuro-muscular transmission show the need for much further investigation. In particular, the physiology of mammalian neuro-muscular transmission is almost an unexplored field, and most of the experiments supporting the acetylcholine hypothesis have been performed on mammalian muscle.

An attempt to investigate systematically the physiology and pharmacology of mammalian neuro-muscular transmission will be reported in this and subsequent papers. Preliminary accounts of some of this work have already been published [Eccles & O'Connor, 1938 *a, b*; 1939, *a, b, c*].

Histologically the problem may be expressed as follows [cf. Eccles, 1936, p. 399]. At the neuro-muscular junction the motor nerve fibre penetrates the sarcolemma and forms an ending of variable complexity in a specialized region of the muscle fibre called the motor end-plate. Histological and degeneration experiments clearly differentiate between the nerve and the end-plate protoplasts, there being no evidence of protoplasmic continuity [Cajal, 1934]. There is general agreement that the end-plate is to some extent at least a specialized region of the muscle fibre, though it appears also to include nuclei homologous with those of the sheath cells of nerve fibres [Couteaux, 1938 *a, b*]. Thus the evidence suggests a clear break in continuity between motor nerve fibres and the

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motor end-plate, but the relationship of motor end-plate to muscle is uncertain, protoplasmic continuity possibly obtaining to some extent. The state of the true surface membranes of the muscle fibre and motor end-plate is unknown, being, on account of its ultramicroscopic character, more a physiological than a histological problem. Thus we have three structures involved in neuro-muscular transmission—the nerve, the motor end-plate and the muscle fibre. The motor end-plate together with the terminal nerve fibres may be called the motor end-organ.

METHOD

Most of our experiments have been performed on cats under nembutal anaesthesia, an initial injection of 35–40 mg./kg. being given intraperitoneally with subsequent small injections during the course of the experiment so as to keep the degree of anaesthesia approximately constant. In the remaining experiments the cats have been decerebrated under deep ether anaesthesia, sufficient time being allowed (at least 2 hr.) for the effect of this short anaesthesia to pass off. However, with this method of preparation, a single nerve volley often sets up a repetitive response of some muscle fibres [cf. Eccles & O'Connor, 1938 *a*], a complication which is only rarely present in cats under nembutal anaesthesia.

The muscles (soleus, tibialis anticus) are isolated by careful dissection, the enveloping fascia being left intact and the blood supply undisturbed. The tendon with a small piece of bone is detached from its insertion, and the muscle is freed from surrounding tissue as far proximally as the point where it begins to arise from the bone. Fixation of the bony origin of the muscle is secured by two drills, one through the distal ends of the tibia and fibula, and the other through the proximal end of the tibia or the distal end of the femur. These drills are rigidly held in the clamps of a Brown-Schuster myograph, and the tendon, through its attached bone, is connected to the myograph lever by a steel hook containing a bakelite intercept for the purposes of electrical insulation. The knife-edge bearing of the mirror myograph has minimized friction.

The motor nerve is isolated and cut centrally (except in a few experiments with a central novocaine block), and all motor branches to other muscles are divided. Two fine loops of thread moistened in saline serve to connect the motor nerve to the stimulating electrodes, which are silver hooks applied to the nerve as close as possible to the muscle and which lift the nerve clear of the surrounding tissues. Break induction shocks from coreless coils are used for stimulation, the primary currents being broken by a Lucas pendulum synchronized with the camera. The

shocks are at least five times stronger than the weakest stimulus which elicits a maximal muscular contraction, and their times are signalled by the stimulus artefacts. In order to prevent any cumulative effects of muscle activity, an interval of at least half a minute has been allowed between successive observations.

For diffuse leading from the muscle, two stout collars of cotton wool soaked in Ringer's solution are placed around it, one at its tendinous end and the other more proximally but also on the isolated part of the muscle. Silver hooks serve to connect these collars respectively to the grid and earthed terminals of a resistance-capacity coupled amplifier which has a time characteristic of 1.9 sec. The output from the amplifier is recorded by a Cossor cathode-ray oscillograph, and the photographic system has already been described [Eccles, 1935*a*]. Photographs are taken with the plates moving at a uniform speed of about 2.0 m./sec., and in the time axis the records can easily be measured under low magnification to 0.05 mm.; hence our time measurements are significant to 0.025 msec. The Lucas pendulum operates with approximately this degree of accuracy in delivering stimuli at chosen intervals apart.

Extraneous electrical disturbances are eliminated by housing the preparation and all the apparatus (entirely battery operated) in an electrically shielded room constructed of galvanized iron sheets soldered together. Ventilation is secured by a draught system operated by an external electric fan, the air being completely replenished every 2 min.

The exposed tissues are kept warm and moist by enclosing them in a bakelite box, heated by three 24 W. lamps, covered in gauze which soaks up water from a subjacent trough. The head of the animal projects from this box and the hook connecting the muscle to the myograph passes through a small hole in the roof (Fig. 3 B). Frequent readings of a thermometer projecting from the box have indicated that, with rare exceptions, the temperature has been maintained steady between 37 and 39° C. With these precautions the exposed nerve and muscle have remained in good condition for as long as 10 hr. The animal's temperature has also been frequently recorded by a thermometer placed deep in its neck, and also has usually been maintained between 37 and 39° C.

A. THE RESPONSE OF MUSCLE TO A SINGLE NERVE VOLLEY

1. *The preparation with a localized end-plate zone*

With the diffuse leads from the belly and tendon of the muscle, as above described, a single motor nerve volley always gives rise to action potentials whose irregularities are evidence of a complex character

(Fig. 1). Since our aim has been to study a muscle as nearly normal as possible and with an intact blood supply, it was not permissible to attempt a simplification of the action potential either by making it monophasic through injury of the distal end of the muscle, or by dissecting a uniform strip from the complex muscle mass. Early in our investigation attempts were made to cut down the motor nerve, but, even when this cutting down was continued so that only a minute fraction of the muscle retained its innervation, the action potential was still complex. This technique was, therefore, abandoned, and our experimental investigations were almost completed before it was again attempted. Then, by chance, a "cut-down" preparation was obtained in

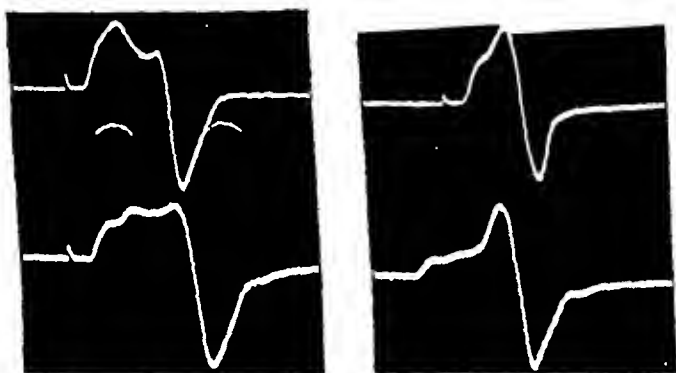


Fig. 1. Typical examples of the complex action potentials set up by a single nerve volley in an intact soleus muscle. Time: 1 d.v. = 10 msec.

which a single nerve volley set up an action potential having a single sharp spike. Investigation with different positions of the electrodes revealed that excitation was confined to a small narrow strip of muscle fibres (Fig. 2) and that the impulses in these fibres all appeared to start at about the same point and at the same time—hence the sharp negative spike obtained when the earthed electrode was applied at any point near to this muscle strip, the grid lead being on the muscle tendon. The few intact motor nerve fibres were all terminating in motor end-organs restricted to a narrow transverse zone on the innervated muscle fibres.

This preparation gave opportunity for the direct investigation of electrical events occurring in the muscle both at the region of its innervation and distal from this region. Subsequent attempts to repeat this dissection have shown that a successful result is best obtained by leaving

intact only a minute twig which arises from the motor nerve at the point where it subdivides into its main branches and penetrates the muscle. This twig should be one which tends to run superficially and which breaks up into branches after a very short course. The innervated muscle fibres will then be found to form a discrete superficial strip whose motor end-organs are restricted to a region close to the site of the nerve entry (Fig. 2). Soleus was particularly suited for such preparations, tibialis

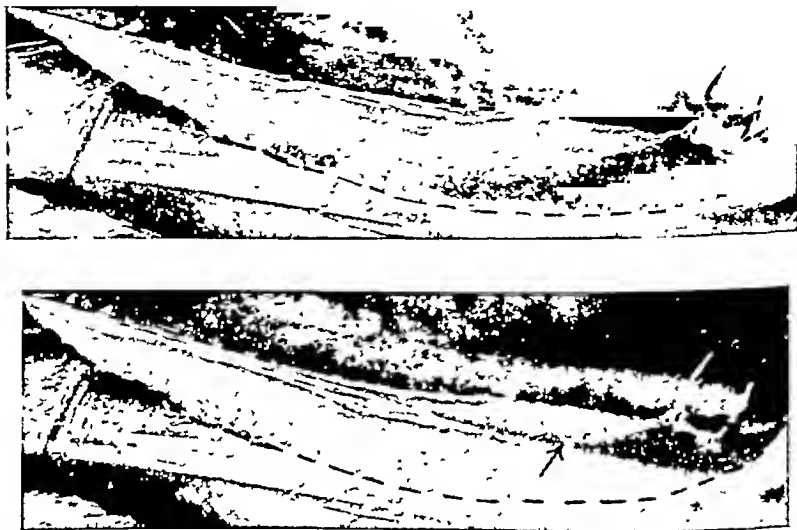


Fig. 2. Photographs of a soleus preparation with innervation restricted to a superficial strip. In the lower picture, the strip is contracting (cf. resting condition shown above) in response to maximal tetanic stimulation of the motor nerve through the electrodes seen on right. The arrow indicates the approximate position of the end-plates on the strip. Distortion of the tissues is much less with contractions evoked by single or double stimuli.

anticus proving unsatisfactory. Fortunately, the small peroneus tertius also gave satisfactory preparations, and in all its properties it closely resembled tibialis anticus. After a little practice we found no difficulty in preparing these muscles with localized motor end-organs, and all our original work on intact muscles has been repeated on our thirty-three successfully dissected preparations. All the nerve dissections are made under observation by a binocular Zeiss dissection microscope, a magnification of 20 diameters being found most suitable. To some extent direct observation of the muscle during a twitch gives an indication of the localization of the muscle innervation (Fig. 2), but the only reliable test



Fig. 3A.

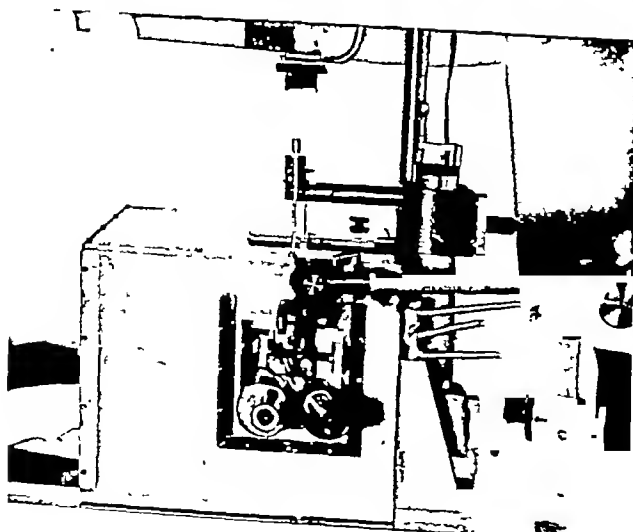


Fig. 3B.

is provided by the muscle action potentials recorded with varying positions of the leads.

As leads we used for the greater part of our investigation fine silver wires running transversely across the muscle, one just touching it for a short distance over the innervated muscle strip, and the other either touching the innervated strip elsewhere or being relatively indifferent and touching the muscle some distance away from the active muscle strip, e.g. the tendon. With such electrodes there is a risk that the silver might act injuriously on the subjacent tissues; hence we now use as an electrode a glass tube ending in a fine terminal hole (about 0.5 mm. in diameter) and filled with Ringer Locke solution into which projects a silver wire coated with AgCl (cf. Fig. 3A). The experiments carried out with this improved type of electrode have been similar in every respect to those with the silver-wire electrodes.

The action potentials recorded with either type of electrode correspond in their main features with the potentials that would be produced by a brief wave of negativity (the muscle impulses) propagated along the muscle fibres. Thus, in Fig. 4A, the short latent period and abrupt rise to the negative spike indicate that the active electrode is applied close to the origin of the muscle impulses, i.e. close to the motor end-organs; while in Fig. 4B and C the delayed triphasic spike shows that the impulses have to be conducted a considerable distance before reaching the active electrode. Now the muscle fibres in which the impulses are propagated form a narrow strip embedded in a conducting medium composed of the inactive (denervated) muscle fibres. Hence the triphasic action potentials (a negative spike between two positive waves, cf. Fig. 4B, C) picked up by one active electrode (on the innervated muscle strip) and one relatively indifferent electrode would be produced by the propagation of a pure wave of negativity along the muscle strip, exactly as occurs with nerve or muscle embedded in an extensive conducting medium. Gilson & Bishop [1937] and Bishop [1937] give an experimental and theoretical treatment of this problem. The initial positive wave is produced before the impulse reaches the active electrode, and the final positive wave after it has passed beyond this electrode. Thus there is no initial positive wave if the active electrode is applied immediately over the end-plate zone, there being then a sharp initial negative spike and a later positive wave (Fig. 4A). Also, if the active electrode is applied over the tendinous endings of the active muscle fibres, the final positive wave is not observed because the muscle impulses are then not propagated beyond the electrode. With these two exceptions each lead

applied to the muscle strip will pick up a triphasic action potential, so six waves are recorded when two such leading electrodes are applied to the muscle strip at a sufficient distance apart (Fig. 5).

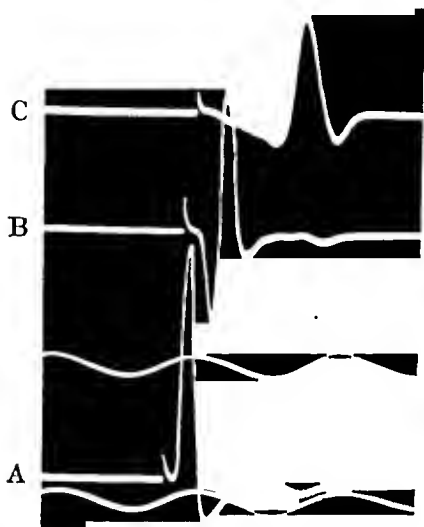


Fig. 4.



Fig. 5.

Fig. 4. A, Action potential set up by a single nerve volley, one electrode being on the end-plate zone of the innervated muscle strip, the other on an inactive region of the muscle. B, C, Action potentials recorded with one electrode on the active muscle strip respectively 4 and 16 mm. distally from the end-plate region, the other being on an inactive region of the muscle. Time: 1 d.v. = 10 msec.

Fig. 5. Action potentials set up by a single nerve volley and recorded when both electrodes are on the active muscle strip distal to the end-plate zone. Time as in Fig. 4.

2. *The conduction velocity of muscle impulses*

Measurements of the latency of the spike crest with different positions of the electrodes have been used in determining the conduction rate of the muscle impulses, and also in locating accurately the region of the motor end-plates. If both electrodes are placed on the active muscle strip at more than 1 cm. apart, an essentially diphasic action potential is obtained with no overlap between the main negative and positive spikes of each diphasic or triphasic complex. These spikes consequently signal the arrival of the crest of the muscle impulse at the respective

electrodes (Fig. 5). Fig. 6 shows the curve obtained by plotting these times against the corresponding positions of the electrodes. For a small region of the muscle—about 2 mm. in length—the latent periods have a constant minimal value, indicating that the great majority of the motor end-plates are distributed over this zone. Beyond this zone in both directions the latent period of the muscle spike shows a similar linear increase with the distance, indicating that the muscle impulses are propagated at a uniform rate from the end-plate zone towards both ends of the muscle. The velocities of propagation so determined have varied from 2.85 to 4.8 m./sec. for soleus muscle and from 4 to 7 m./sec. for peroneus tertius. These measurements from spike crests would give values for the modal class of propagation velocity for the population of active muscle fibres. Accurate measurements of the fastest conduction velocity cannot be made on account of the complications introduced by the triphasic nature of the action potentials, but the action potential shapes indicate that there is but little increase in temporal dispersion with propagation, so the fastest values are probably but little more, and the slowest but little less, than the modal value.

3. *The neuro-muscular delay*

Fig. 7 (cf. also Fig. 4 A) shows examples of the action potential evoked in a soleus muscle by a single nerve volley when one electrode is applied to the motor end-plate zone and the other is far removed from the activated muscle strip. In our experiments on soleus the motor nerve volley has been set up by a break induction shock with the cathode of the stimulating electrodes 6–9 mm. from the termination of the nerve fibres at the motor end-plates, and the latent period from the stimulus to the beginning of the action potential has varied from 0.75 to 0.85 msec. This latent period is observed even with greatly increased amplification (Fig. 8), so it provides a true measure for the time of onset of electrical changes at the end-plate region of the muscle. Records with high amplification, however, may show an earlier, very brief action potential superimposed on the latter part of the stimulus artefact, and this presumably is the nerve action potential. But it is not justifiable to assume that this action potential signals the time of arrival of the nerve impulses at the motor end-plates, for it will be produced by these impulses as they traverse the whole of their short intra-muscular course. The actual time of arrival of the nerve impulses at the motor end-plates cannot, therefore, be directly measured.

Attempts at calculation of this time of arrival are subject to a considerable uncertainty on account of the profuse branching of the motor nerve fibre as it nears its destination in the motor end-organ; for each nerve fibre on the average innervates about 150 muscle fibres

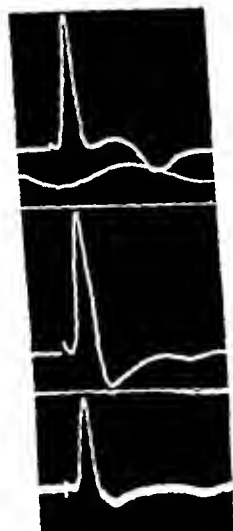


Fig. 7.

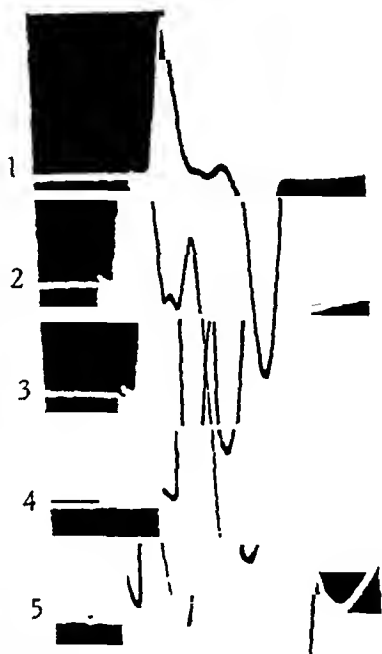


Fig. 8.

Fig. 7. Action potentials set up at the end-plate zone by a single nerve volley in three experiments. One electrode is on the end-plate zone, the other on an inactive part of the muscle. Time: 1 d.v. = 10 msec.

Fig. 8. Action potentials set up at the end-plate zone by a single nerve volley, one electrode being on the end-plates, the other on a distal part of the active strip. With observation 1 the whole course of the action potential is seen. The amplification is progressively increased in observations 2, 3, 4 and 5, being respectively 6.5, 15, 36 and 84 times that of observation 1. Measurements of the latent periods of the action potentials show only a slight shortening (about 0.05 msec.) even with this great increase in amplification. Time scale as in Fig. 12.

[Clark, 1931]. With each subdivision the daughter fibres are smaller than the parent fibre, though the combined cross-sectional area is usually slightly larger than that of the parent fibre [Eccles & Sherrington, 1930]. The repeated diminution in size which accompanies the branching of the motor nerve fibres must result in a slowing of conduction of the motor

nerve impulses [cf. Erlanger & Gasser, 1937, pp. 38-40]. Thus, if the impulse conduction velocity is taken to equal that for the unbranched length of the motor fibres (probably 60-80 m./sec. for the rather small motor fibres in soleus), the conduction time will be underestimated. Certainly the nerve conduction time will be at least twice this calculated value, i.e. at least 0.2 msec., but it may be considerably longer, so our calculation only gives an upper limit of about 0.55-0.65 msec. for the shortest neuro-muscular delays. Further evidence relating to the durations of the neuro-muscular delays and to the extent of the range from the shortest to the longest delays will be given in a later paper (see also section A, 4).

With peroneus tertius muscle there is a similar upper limit (0.55-0.65 msec.) for the shortest neuro-muscular delays, and experiments on intact tibialis anticus also give similar values (0.6-0.7 msec.). These values are in good agreement with Lorente de N6's [1935] figure of 0.6 msec. for the neuro-muscular delay of the mammalian extrinsic eye muscles.

4. *The muscle action potential*

The brief latent period of Figs. 4A, 7 and 8 is followed by an abrupt development of negative potential at the end-plate electrode, the crest being attained in about 0.75 msec., and the decline occurring in rather less than twice this time. The total duration of the negative spike is thus about 2 msec. in the experiments illustrated in these figures. A few of our soleus preparations have given action potentials having a total duration of even more than 3 msec. In some of these experiments at least, this longer value has been due to a more diffuse location of the activated motor end-plates, for the duration of the negative spike has been shortened to about 2 msec. by further pruning of the intact motor nerve twig. An impulse in a single muscle fibre should give a spike response even shorter than 2 msec., for some asynchronism of the impulses which a single nerve volley sets up under the end-plate electrode must occur both on account of some disparity in their individual latent periods, and also on account of the short conduction distance to the electrode from some motor end-plates even with our most circumscribed groups of activated end-plates. The brevity of the spike indicates that the asynchronism must be small, hence there can be but a small disparity in the individual latent periods, 0.5 msec. possibly representing the upper limit thus indicated for this disparity. We have seen above that 0.55-0.65 msec. is the upper limit for the shortest neuro-muscular delays; hence the greatest possible disparity indicates that the longest

neuro-muscular delays can be little, if any, longer than 1 msec. in duration.

If 2 msec. be taken as the duration of the muscle spike, and 3.5 m. as the conduction velocity, then at any instant the muscle impulse will extend for about 7 mm. along a soleus muscle fibre—no more than one-fifth of the length of a nerve impulse in a mammalian motor nerve fibre [cf. Gasser & Grundfest, 1936]. The rising phase would be about 2.5 mm. in length and the decline about 4.5 mm.

With peroneus tertius the spike action potential is still sharper than with soleus, the crest being attained in 0.45–0.6 sec. and the total duration being 1.4–1.6 msec (Fig. 9). Here the whole range of variation of the neuro-muscular delays must be considerably less than 0.5 msec., the longest delays being certainly less than 1 msec. The much longer value of 10 msec. has been given by Rosenblueth & Morison [1937] for the longest neuro-muscular delays of the mammalian facial muscles.

Taking 1.5 msec. as the total duration of the spike of peroneus tertius and the average value of 5 m./sec. as the conduction velocity, the muscle impulse must extend about 7.5 mm. along a peroneus tertius muscle fibre—a value very similar to that derived above for soleus. It may be noted that, at any instant, nerve impulses also extend for about the same length (about 4 cm.) along nerve fibres, even though they vary widely in their conduction velocities [Gasser & Grundfest, 1936].

The simple spike potentials of muscles whose motor end-plates are restricted to a localized zone show that the complex and prolonged spike potentials recorded from the whole muscle (Fig. 1) must usually be entirely due to the asynchronous arrival of the muscle impulses at the two leading electrodes, and that there is only one impulse in each muscle fibre. Some muscle impulses will arise at motor end-plates just beneath one leading electrode and immediately give their spike potential—other muscle fibres will have their end-plates far removed from a leading electrode, a spike potential being recorded from them only after a considerable muscle impulse conduction time. The observed values for muscle-impulse velocity and for the length of the muscle fibres from their end-plates to their ends is adequate to account for the asynchronism (as much as 10 msec. with soleus) of the spike potentials recorded from the whole muscle. Usually the spike potentials show an asynchronism no longer than this, but with some muscles a still larger temporal dispersion of muscle impulses is produced by a repetitive discharge of impulses from the motor end-plates [cf. Eccles & O'Connor, 1938*a*]. These experiments will be fully dealt with in a later paper. Such repetitive discharges do



Fig. 9A. Action potentials set up at the end-plate zone of peroneus tertius by two nerve volleys at the intervals approximately indicated by the white dots just above or below the stimulus artefacts. One electrode was at the end-plate region and the other on an inactive part of the muscle.

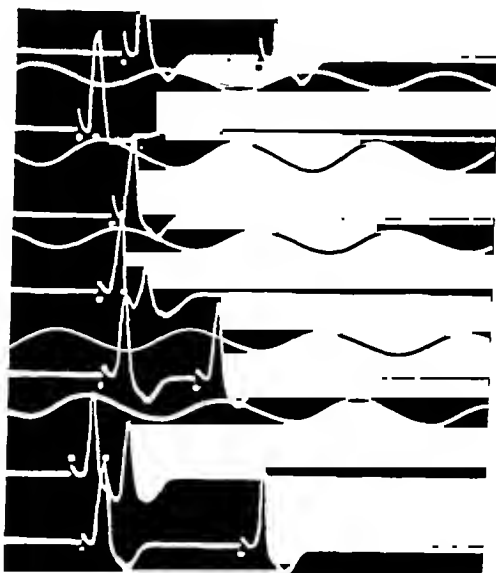


Fig. 9B. As in Fig. 9A in another experiment. Time: 1 d.v. = 10 msec.

not often occur in animals under nembutal anaesthesia, which for this reason was employed in most of the present series of experiments on muscles with localized end-plates.

B. THE RESPONSE OF SOLEUS MUSCLE TO TWO NERVE VOLLEYS

1. *The mechanical response*

In Fig. 10 are plotted a typical series of observations on the maximum contractions of soleus produced when two stimuli (at least five times maximal strength) are applied to the motor nerve at varying intervals apart, the maximum contraction tensions being plotted as ordinates and the corresponding stimulus intervals as abscissae. The curve so plotted will be called the contraction-interval curve. It will be seen that, until

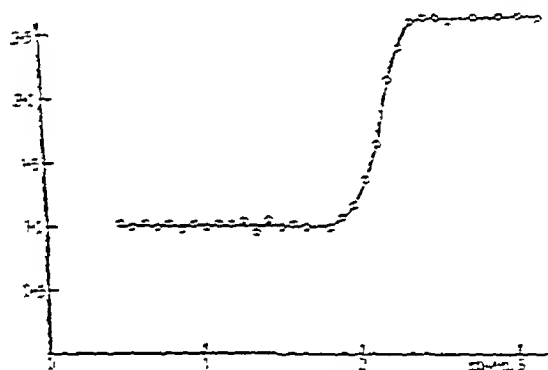


Fig. 10. The maximum contractions of soleus evoked by two nerve volleys are expressed as fractions of the maximum contraction evoked by a single nerve volley and are plotted as ordinates against the corresponding stimulus intervals as abscissae. The stimuli applied to the nerve were seven times the just maximal strength.

the stimulus interval is lengthened to 1.9 msec., the second stimulus fails to increase the maximum contraction tension which is produced in response to the first stimulus alone. This interval of 1.9 msec. must be much longer than the absolutely refractory period of the soleus motor nerve fibres. Toss Gasser & Grundfest [1936] and Graham & Lorente de Nô [1936] showed that the shortest absolutely refractory period in mammalian nerve was only 0.4-0.5 msec. However, a somewhat longer value would be expected for the rather small motor nerve fibres innervating soleus [cf. Eccles & Sherrington, 1930]; later (section B, 2(b)) it will be shown that a second nerve volley is set up and actually excites the motor end-plates when the stimulus interval is as short as 0.7 msec., the stimuli being at least five times maximal. At stimulus intervals less than



Fig. 9A. Action potentials set up at the end-plate zone of peroneus tertius by two nerve volleys at the intervals approximately indicated by the white dots just above or below the stimulus artefacts. One electrode was at the end-plate region and the other on an inactive part of the muscle.



Fig. 9B. As in Fig. 9A in another experiment. Time: 1 d.v. = 10 msec.

applied to the nerve from 0.95 to 2.4 msec. after two volleys at 1.75 msec. apart (Fig. 11 A), it increases the contraction just to the plateau height. Presumably the nerve volley set up by this third stimulus evokes a response in all those muscle fibres that had not previously responded to the second volley; thus the muscle fibres have now two chances for a second response—if the second volley is too early, then the third volley fires them off. However, when the interval between the first and second volleys is 2.4 msec., the third stimulus fails to increase the contraction until separated from the second by 2.0 msec. (Fig. 11 B), an interval sufficiently long to allow it to set up a third response in muscle fibres which have already responded to both the first and second volleys. A triple response of many fibres also gives the late rise which occurs in Fig. 11 A when the third stimulus is more than 2.4 msec. after the second. In Fig. 11 B the failure of the third stimulus to produce an early step (as in Fig. 11 A at intervals as short as 0.65 msec. after the second stimulus), proves that all the muscle fibres had responded to the second volley and, as a consequence, there is the observed delay before a third response could be elicited [cf. Forbes, Ray & Griffith, 1923].

Hence it may be concluded that the plateau of Figs. 10 and 11 is due to the second volley setting up a response in every muscle fibre. The decline from this plateau with volley intervals lengthened beyond 40 msec. is a feature of the wave summation of two maximal responses of muscle [cf. Fulton, 1926; Cooper & Eccles, 1930], and does not signify the failure of the second volley to excite the muscle at such long stimulus intervals. Thus, in Fig. 10, as the stimulus interval is progressively lengthened from 1.9 to 2.4 msec., all of the population of muscle fibres is brought to respond a second time. That part of the contraction-interval curve expressing this progressive involvement appears to approximate to the S-shaped type of population distribution curve, and this suggests that, in their response to two nerve volleys, the population of muscle fibres is symmetrically distributed about a mean critical volley interval of about 2.2 msec. The above arguments neglect the possibilities that some muscle fibres are not excited by a single nerve volley or by the second of two nerve volleys. These possibilities will be excluded in a later paper. For the present only the population of muscle fibres which responds to single and double nerve volleys is under discussion.

With soleus muscles prepared with localized end-plates, similar simple S-shaped contraction-interval curves have been obtained in fifteen of our seventeen experiments, but the finer details of the curves have not been worked out in all of these experiments. In some experi-

about 2 msec. [cf. Graham & Lorente de N6, 1938], the second nerve volley will be travelling more slowly than the first, hence the intervals between the arrivals of the successive volleys at the motor end-plates will be longer than the stimulus interval. The extent of this lengthening cannot be directly determined, but in section E2 indirect evidence will be presented.

Further progressive lengthening of the volley interval beyond 1.9 msec. results in a rapid increase in contraction, until, with intervals

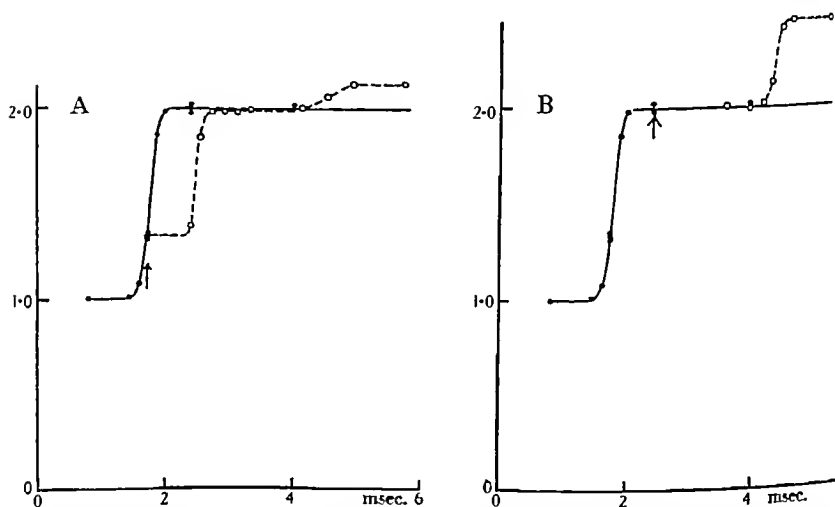


Fig. 11A. The continuous curve and the solid circles are observations from another experiment plotted similarly to those of Fig. 10. The open circles show observations with three nerve stimuli, the second 1.75 msec. after the first (time shown by the arrow) and the third at the plotted interval after the first.

Fig. 11B. As in Fig. 11A, but with the 1-2 interval constant at 2.4 msec. as shown by arrow.

from 2.4 to 3.2 msec., it attains a constant value at a tension 2.6 times the response to a single volley. This suggests that, with a volley interval of 1.9 msec., second impulses are set up in a few muscle fibres, and with longer volley intervals more and more respond. Finally, the plateau or phase of constant tension with volley intervals from 2.4 to 3.2 msec. (a decline is not observed till the volley interval is increased beyond 40 msec.) suggests that all the muscle fibres are responding to the second volley.

This interpretation of the contraction-interval curve may be tested by the application of a third stimulus. When this third stimulus is

impulses in the muscle fibres. A further small lengthening of the volley interval to 2.5 msec. results in the production by the second volley of a spike potential which is almost as large as the first spike potential. Detailed plotting of this increase in spike potential against the corresponding volley intervals from 1.8 to 2.5 msec. (Fig. 21, curve 5, shows just a few points) gives an S-shaped curve closely resembling the contraction-interval curve (Fig. 21, curve 6) plotted as in Fig. 10 for this same series of volley intervals. This agreement is, of course, to be expected, because both S-shaped curves plot the progressive increase in the number of muscle fibres in which the second volley sets up impulses.

In section B1 it was suggested that the strong stimuli we have employed would set up impulses in the fastest soleus nerve fibres even when only 0.7 msec. apart. This is confirmed by experiments such as that illustrated in Fig. 11A, where a third stimulus applied only 0.65 msec. after the second set up impulses in some motor nerve fibres, for it caused some muscle fibres to be excited; and a third stimulus 0.95 msec. after the second must have excited all the motor nerve fibres, for it evoked a contraction response in all muscle fibres not excited by the second volley. Hence, in our experiments, there is a period from about 0.7 msec. to as long as 1.8 msec. after the first volley, during which a second nerve volley fails to set up a propagated impulse in any muscle fibre. And all muscle fibres do not respond to this second volley until the volley interval is as long as 2.5 msec. This failure of the second volley cannot be ascribed to a diminution in its size by the relatively refractory period following the first volley, for, as noted above, a third volley, only 0.95 msec. after the second, was sufficiently powerful to excite all the muscle fibres not responding to the second volley. Moreover, with volley intervals as long as 2.0 msec., the second nerve volley must be travelling in nerve which has almost completely recovered from the relatively refractory state and so its component impulses must be almost full-sized. Hence it may be concluded that, with short volley intervals, the failure of the second nerve volley to set up propagated impulses in the muscle is due to the relatively slow recovery of the muscle after its previous response. However, we shall now see that this failure does not mean that an early second nerve volley produces no response at the region of the neuro-muscular junction.

(b) *The electrical response at the end-plate zone.* Fig. 12 shows a typical series of action potentials recorded with one electrode on the localized end-plate zone of the muscle, and the other on the active muscle strip 22 mm. distally. Since this distal electrode was on the muscle fibres close to their tendinous endings, it only picked up a

ments the decline from the plateau contraction was observed with volley intervals as short as 10 msec. With two exceptions the critical stimulus interval for an increase in contraction has varied from 1.8 to 2.2 msec., the mean critical stimulus interval from 2.0 to 2.3 msec., while the plateau has been attained at intervals varying from 2.1 to 2.5 msec., the temperature of the muscle lying in all cases between 37 and 38.5° C. In the two exceptional experiments the respective intervals were 1.4, 1.6 and 1.8 msec. The ratio of the plateau tension of the double response to the maximal twitch tension has usually varied from 1.9 to 2.5, but larger values (up to 6.0) were obtained with very small innervated muscle strips in three of our experiments.

Almost identical figures have been obtained in the six of our eighteen experiments on intact soleus muscles in which a simple symmetrical S-shaped contraction-interval curve has been obtained, the respective intervals as given above being 1.5–1.8 msec., 1.8–2.0 msec., and 2.0–2.4 msec., while the ratio of double to single response has varied from 1.8 to 2.05. These experiments were performed on decerebrate animals which had recovered completely from brief ether anaesthesia, hence the much higher proportion of complicated contraction-interval curves. In a later paper such complicated responses will be shown to be caused by the repetitive discharge of impulses from the motor end-plates [cf. Eccles & O'Connor, 1938*a*]. In the series of experiments with localized end-plates, this complication was avoided in all but two experiments by working on intact animals under nembutal anaesthesia. The very high ratios of double to single contraction sometimes observed in the muscles with localized end-plates is presumably due to the damping effect of the large inactive muscle mass on the tension development of a single twitch, this effect being less on the larger and longer contraction produced by the double twitch.

2. *The electrical response*

(a) *The electrical response distal from the end-plate zone.* When muscles are prepared with their innervation restricted to a localized group of motor end-plates, the electrical responses evoked by a second nerve volley may be observed either at the region of these end-plates, or distal from this region. Recording distal from the end-plates, e.g. with one electrode on the tendon and the other on the innervated muscle strip more than 1 or 2 mm. from the localized end-plate zone, the second volley adds nothing to the potential produced by the first volley until the volley interval is lengthened beyond about 1.8 msec. Therefore, with these short volley intervals, the second volley fails to set up propagated

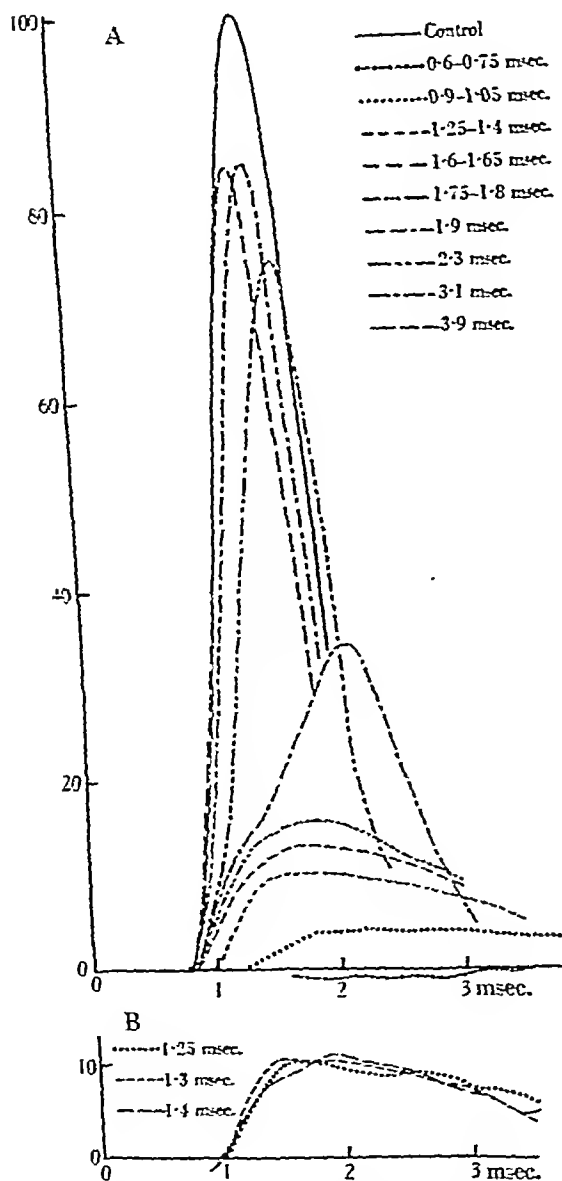


Fig. 13. A, Time courses of the potential changes, expressed as a percentage of the maximum spike potential, produced by a second nerve volley at the indicated intervals after the first volley. These curves are calculated, as described in the text, from a series of observations which are partly illustrated in Fig. 12. The continuous line shows the spike potential set up by a single nerve volley. B, The three individual curves which are averaged to give the curve labelled 1.25-1.4 msec. in Fig. 13A.

diphasic response, becoming first positive and then negative to the end-plate electrode, as shown by the second phase of the action potential recorded in observation 1 of Fig. 12 (cf. section A1). The action potential of observation 7 (volley-interval 0.7 msec.) does not differ significantly from that produced by a single volley (observation 1). But as the volley interval is progressively lengthened to 0.95, 1.3 and 1.6 msec. (observations 6, 5 and 4 respectively), the second nerve volley is seen to increase the negative potential of the end-plate region relative to the rest of the muscle, this effect being most obvious during and just after the trough following the initial negative spike. The restriction of this additional negative potential to the end-plate region is shown by the absence (section B 2 (a)) of any such potential when the recording electrode is moved more than about 2 mm. from the end-plate zone (see Fig. 6). Finally, at 1.9 msec. interval (observation 3), the second volley adds a small spike potential and at 2.3 msec. (observation 2) this spike potential is quite large. The spike potentials observed in these two latter observations closely agree with those recorded distal from the end-plate zone, and hence must be produced by propagated muscle impulses. In observations 3 and 2 these propagated impulses set up by the second volley give the later complex response recorded by the electrode distal from the end-plate zone (cf. the second diphasic part of the action potential set up by a single nerve volley

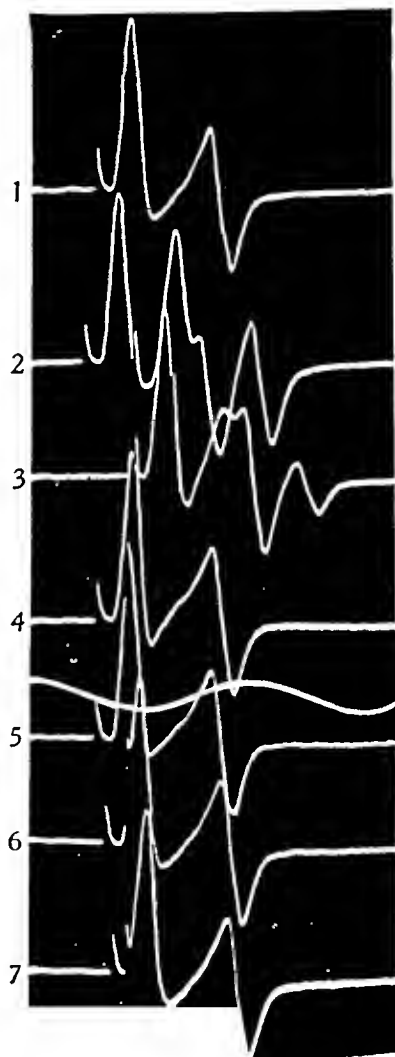


Fig. 12. Action potentials set up in soleus muscle by single or double nerve volleys and recorded by one electrode on the end-plate region and one 22 mm. distally on the innervated muscle strip. Further description in the text. Time: 1 d.v. = 10 msec.

in observation 1). On the other hand, this distal electrode picks up no trace of such a propagated potential in observations 4, 5 and 6; hence the negative potential produced by the second volley at the end-plate zone cannot be due to propagated muscle impulses—a conclusion thus derived from both the potential-interval curves recorded away from the end-plate zone and the contraction-interval curves (section B1).

The time course of the negative potential added by the second nerve volley at the end-plate region has been determined by subtracting the response to the first volley from that to the two volleys, the subtracted curves so obtained being plotted with the times of the respective second stimuli synchronized at zero. With short volley intervals the added negative potentials have been so small that our error in measurement has produced considerable irregularities in the plotted curves. To some extent these random irregularities have been eliminated in Fig. 13A by averaging several curves which were obtained with approximately the same stimulus intervals, and which did not show any significant systematic deviations from one another. Thus the curve of Fig. 13A for volley intervals of 1.25–1.4 msec. is obtained from the three curves shown in Fig. 13B for volley intervals of 1.25, 1.3 (shown in observation 5, Fig. 12), and 1.4 msec. The added potential shows an earlier beginning and an increased size as the volley interval is lengthened from about 1.0 msec. to 1.25 msec., but only a small change is observed for the lengthening from 1.25 to 1.4 msec. (Fig. 13B). However, further lengthenings of the volley interval to 1.6–1.65 msec., and to 1.75–1.8 msec., again give an increased potential wave with a still earlier beginning.

In Fig. 12 the large secondary spike potentials set up by the first nerve volley and arising as the muscle impulses reach the distal electrode prevented an accurate determination of the negative potential added by the second volley after about 3 msec. In some experiments this interference has been minimized by placing the distal electrode well away from the active muscle strip, i.e. by making it relatively indifferent (Fig. 14). The later stages of the added negative potential could then be determined by subtraction as for Fig. 13, observations from one experiment being shown in Fig. 15.

With volley intervals of 1.0 and 1.36 msec., the added negative potentials are seen to be almost identical, the only significant difference being a slightly earlier origin of the 1.36 msec. response. The summit is attained at about 2.5 msec. after the setting up of the second nerve volley, the decline to half about 2.5 msec. later, while at 10 msec. very little potential remains. With a volley interval of 0.87 msec., the added



Fig. 14. Action potentials set up as in Fig. 12, but recorded with one electrode on the end-plate region and one on an inactive part of the muscle. Observation 6 gives the action potential evoked by a single nerve volley. A second stimulus at 0.55 msec. gives no additional potential (observation 2), but with intervals of 1.6, 1.8, 1.4, 1.9 and 1.2 msec. (observations 1, 3, 4, 5 and 7 respectively) an additional slow negative potential is set up by the second volley. This volley, however, fails to set up either a muscle spike potential or an additional muscle contraction. Time: 1 d.v. = 10 msec.

and consequent hump-like shape indicates that this increase observed with intervals longer than 1.36 msec. is due to a different process from that giving the negative potential at the shorter volley intervals; hereafter the term "hump-like" will be used to designate such additions. Simultaneous observation of the muscle contraction shows that the second nerve volley at 1.83 msec. has failed to produce any additional response (Fig. 16), so it must be concluded that the hump-like addition to the negative potential is not due to the setting up of fully propagated muscle impulses by the second volley. Now at the slightly longer volley interval, 1.93 msec., the second volley gives a much larger hump-like response and the large additional muscle contraction (Fig. 16) shows that it has set up fully propagated impulses in about one-third of the muscle fibres. The electrical response confirms this inference, because the trough which follows the hump would be produced by impulses as they passed along the muscle fibres beyond the end-plate zone (cf. the response to a single volley in Fig. 15, and see also section A1). The absence of fully propagated muscle impulses at 1.83 msec. volley interval is confirmed by the absence of any trough-like depression after the hump.

The effect of volley interval on the negative potential set up by the second nerve volley may be illustrated by plotting one against the other, e.g. Fig. 17 A gives the complete series of observations which are in part illustrated in Fig. 15. Such a potential-interval curve as Fig. 17 A shows that lengthening of the volley interval from 0.7 to 1.0 msec. is associated with an increase in the negative potential to a plateau from which there is a further increase, first detectable at volley intervals just beyond 1.3 msec., and finally sharply ascending at those volley intervals where fully propagated impulses are set up by the second volley. However, the beginning of this steep ascent is due not to propagated impulses but to the hump-like addition, as is clearly shown in Fig. 15 by the observation at 1.83 msec. interval (cf. Fig. 17 A). Similar series of observations are shown in Figs. 17 B and C.

On the other hand, Figs. 17 D, E, illustrate a different type of potential-interval curve. Here there is, as above, the steep initial ascent to a plateau from which at the respective intervals of about 1.1 and 1.3 msec. there is a further rise to a second plateau which finally turns up to the steep ascent at intervals of about 1.65 and 2.0 msec. respectively. The time courses of the negative potentials produced by the second volleys (Fig. 18) show that the increased potential of the second plateau is due to an increase in the whole course of the negative potential and not to a hump-like addition. Thus. in these experiments, we have a double

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negative potential is smaller throughout the whole of its course. With volley intervals longer than 1.36 msec. there is a further increase in the added negative potential, which Fig. 15 shows to be quite large at

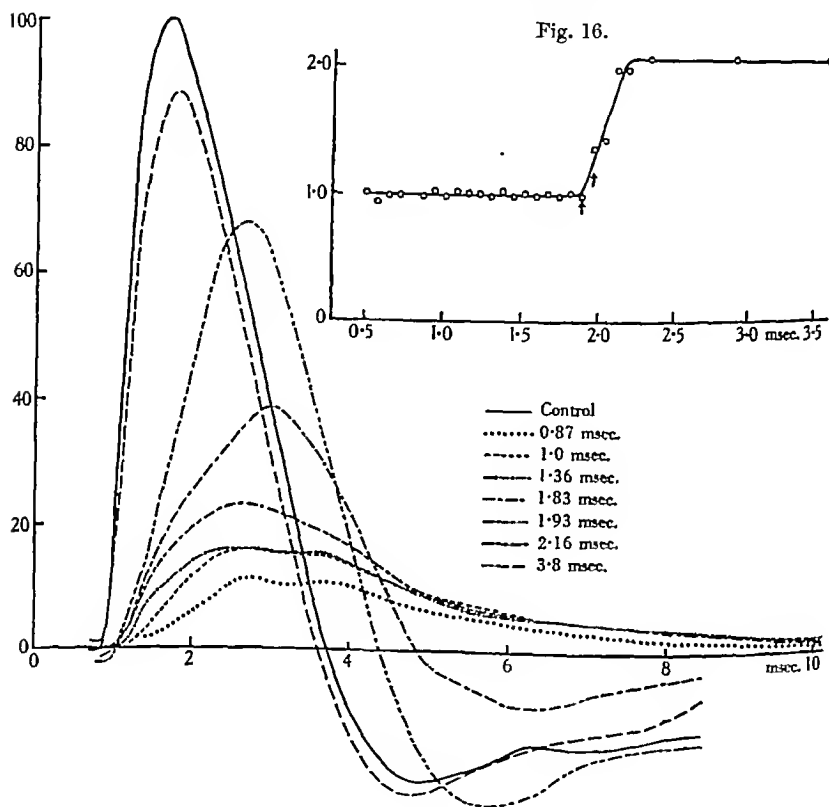


Fig. 15.

Fig. 15. A series of curves obtained as in Fig. 13 from observations resembling those of Fig. 14, but taken from another experiment. The shorter duration of the increase when the interval is lengthened from 1.36 to 1.83 msec. is well shown; this is the hump-like increase discussed in the text.

Fig. 16. Contraction-interval curve (plotted as in Fig. 10) for the series of observations partly shown in Fig. 15. The arrows indicate the observations at 1.83 and 1.93 msec. intervals.

1.83 msec. This increase is solely in the early part of the added potential, for beyond 4.5 msec. the course of the added negative potential does not significantly deviate from that observed for volley intervals of 1.0 and 1.36 msec.; the short duration of this further increase gives it a characteristic hump-like appearance well shown in Fig. 15. Its shorter duration

increase in a potential response whose similar time course suggests that it is not of composite character. This will be further considered in section B2 (e) when additional evidence is available. The final steep ascent in Figs. 17D, E, would seem to be identical with that in Figs. 17A, B, C, the hump-like addition here also forming a transitional phase to the very steep ascent which is due to the rapid increase in the propagated impulses with further slight lengthenings of the volley interval. It may be noted that in Fig. 17A there is also a suggestion of a rise to a second plateau, but the slightness of this rise prevents us from determining if it is due to an over-all increase in the negative potential or to a hump-like addition to it.

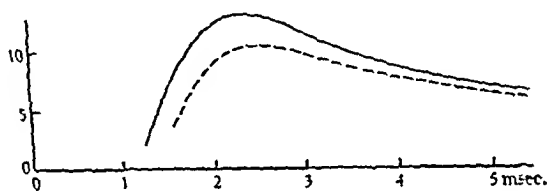


Fig. 18. Time courses (plotted as in Fig. 13) of the potential changes produced by the second nerve volley in some of the observations plotted in Fig. 17E. The lower curve is the average of three closely similar curves of the first plateau of Fig. 17E, and the upper curve of three closely similar curves of the second plateau.

Similar but less extensive series of observations have also been made in ten other experiments, some conforming to the type illustrated in Figs. 17D, E, others to that of Fig. 17B, others possibly transitional as in Fig. 17A. Thus it may be concluded that when the "refractoriness" of the muscle prevents the setting up of propagated muscle impulses, a nerve volley may still set up two kinds of negative potential at the region of the motor end-plates. One is a smooth potential wave reaching, in our fifteen experiments, a rounded summit in 2 to 2.5 msec. and declining to half in 4-6 msec. after the calculated time of arrival of the nerve impulses at the motor end-plates. The other is a much briefer hump-like potential, which has a time course resembling that set up by fully propagated impulses in the muscle fibres. It is only detectable with certainty at volley intervals just shorter than those at which fully propagated impulses are set up. Thus it forms a transitional condition between the responses consisting entirely of the above smooth negative potential wave and those showing the sharp muscle spike produced by the fully propagated muscle impulses.

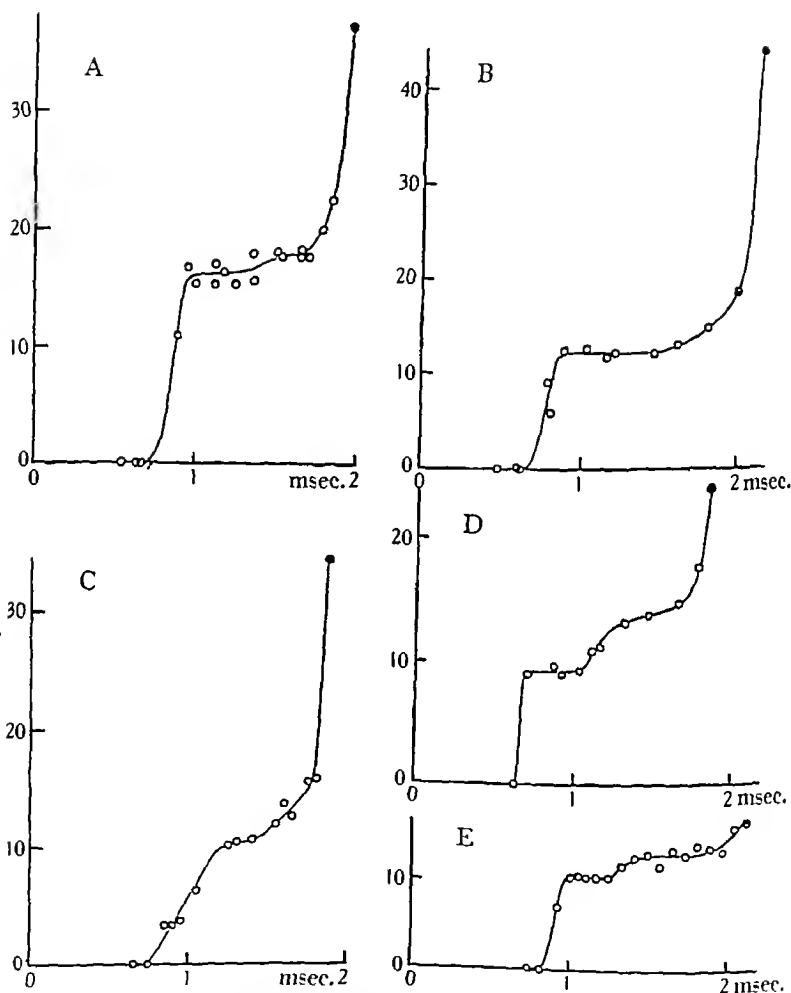


Fig. 17. The maximum negative potentials set up in response to the second stimuli are calculated as percentage of the maximum spike potential as in Fig. 13, and are plotted as ordinates against the corresponding stimulus intervals as abscissae. Curves from five separate experiments are shown. The solid circles are responses in which the second stimulus evoked the discharge of some fully propagated impulses from the motor end-plates. A few such impulses were also probably discharged in the two observations (one in 17B and the other in 17E) which are shown by a horizontal line crossing the open circle. In all other observations there was no evidence of such fully propagated impulses either in our electrical or in our simultaneous mechanical observations.

Bremer [1938] that these negative potentials are produced in the nerve cells with which the synaptic contacts are being made. This explanation must presuppose that the negative potential is produced by the cells even when the synaptic stimulation is inadequate to set up the discharge of impulses therefrom, and thus encounters no difficulty from the recent observations of Fessard & Matthews [1939], who show that these potentials are produced in the spinal cord when a single nerve impulse enters by a dorsal root fibre.

So far we have made only preliminary observations into the possibility of electrotonic transmission of the smooth negative potential wave from the regions of the neuro-muscular junctions out along the motor nerve fibres. In three experiments both leads have been on the isolated motor nerve, one being just at its point of entry into the muscle and only about 3 mm. from its termination in the circumscribed group of motor end-plates. With this method we have failed to detect any electrotonic propagation of the potentials produced at the region of the neuro-muscular junction. In five experiments we have compared the action potentials recorded with the following disposition of the leading electrodes. One is kept fixed on the end-plate zone and the other is either on the nerve at the point of its entry into the muscle or on the muscle itself, this latter electrode being either distally on the innervated muscle strip (cf. Fig. 12) or on some non-innervated region of the muscle (cf. Fig. 14). In three of our experiments, with one lead on the point of nerve entry, the smooth negative potential wave has borne a larger ratio to the muscle spike potential than is obtained with this lead in any position on the muscle. In fact, a similar ratio is observed with any position of the "indifferent" lead on the muscle, whether on the innervated muscle strip or not. Thus, under certain conditions, the nerve is capable of acting as a selective lead for the potentials produced at the end-plate region, its effect being perhaps detectable only when the innervated end-plates are mostly situated on muscle fibres below the surface layer. Under such conditions electrotonic transmission along the nerve fibres might aid our superficially situated electrodes in picking up potentials produced at the region of the motor end-plates, the smooth negative potential thus being increased relative to the muscle spike. The electrotonic transmission of such a potential along the motor nerve fibres does not show that it is produced in the terminals of these nerve fibres, for the potential set up in the spinal cord by a dorsal root volley is transmitted electrotonically along an adjacent dorsal root almost as effectively as along the root of entry [Barron & Matthews, 1938].

Discussion. Rushton [1937] has recently shown that, on the electrical theory of the nerve impulse, excitation of a length of nerve fibre less than a critical value would result in a decremental propagation of an impulse terminating in its rapid extinction. These theoretical deductions have been fully confirmed experimentally by Hodgkin [1938] on isolated single fibre preparations of crab's nerve, subthreshold stimuli of more than half-threshold intensity setting up "new-born" impulses which fail to grow up into fully propagated impulses. Such "new-born" impulses which die out quickly would also be expected to be produced in muscle fibres by stimuli just too weak to set up fully propagated muscle impulses, and the hump-like potential observed at 1.83 msec. interval in Fig. 15 corresponds in every respect with the potential which such "new-born" impulses would set up. Thus, on account, presumably, of the refractory state of the muscle fibres subsequent to the first response, this second nerve volley only sets up "new-born" impulses which are too small to grow up into fully propagated impulses, these abortive impulses giving the hump-like response.

Fig. 17 shows that as the volley interval is shortened below about 1.6 msec. the negative potential declines to a plateau. Here the second volley produces a negative potential uncomplicated by any hump-like addition. Presumably the refractoriness of the muscle is now so great that the second nerve volley fails altogether to set up new-born impulses, the condition corresponding to that observed by Katz [1937], Hodgkin [1938] and Ledingham & Scott [1938] for nerve stimuli below half threshold intensity. Finally, at still shorter stimulus intervals of less than 0.8–1.0 msec., the second stimulus gives rise to less and less negative potential until it fails altogether, this decline often occurring in two steps.

If the hump-like potential is thus due to newborn impulses, the site of its origin is in the muscle fibres themselves in the immediate neighbourhood of the motor end-plates. The smooth negative potential wave has also been shown to arise at the region of the neuro-muscular junction, so there are three possible sites of its origin: the motor nerve terminals; the motor end-plates themselves; the muscle fibres in the immediate neighbourhood of the motor end-plates. An origin in the motor nerve terminals would correspond with the suggestion of Barron & Matthews [1938] that the negative potentials associated with synaptic transmission between neurones arise in the nerve terminals which are making the synaptic contacts. However, it has been shown [Eccles, 1939] that Barron & Matthews' own experiments render such an explanation most improbable, and strongly support the alternative suggestion of Bonnet &

muscle. With Fig. 19A the deviation is first observed beyond 1.4 msec. Finally, in both figures the deviation becomes still more marked as the

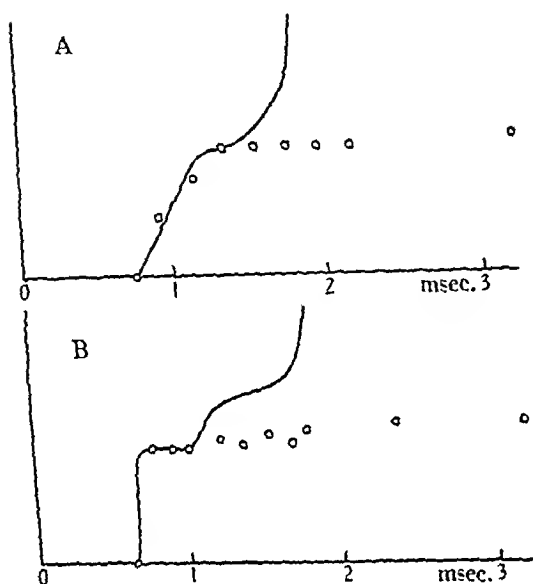


Fig. 19.

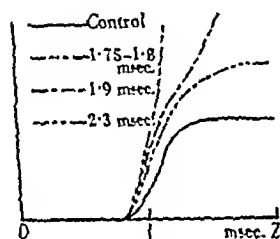


Fig. 20.

Fig. 19. A, The line is drawn as for the series of observations plotted in Fig. 17C, and the plotted points are for a similar series of observations taken after such a deep curarization of the muscle that only end-plate potentials are produced by a nerve volley. The scale of the ordinates has been adjusted so that the plateau for the curarized observations is at the same height as the imperfect plateau observed for the normal muscle in Fig. 17C. B, As in Fig. 19A, but for the experiment illustrated in Fig. 17D. Here the curve for the end-plate potentials in the completely curarized muscle shows an initial plateau giving place to a small ascent at longer volley intervals, but this effect is much less than in the normal muscle (Fig. 17D).

Fig. 20. The earlier parts of three potential curves are taken from Fig. 13, and the continuous line shows approximately the true end-plate potential which, according to the argument in the text, is set up by the second nerve volley at these stimulus intervals. The plotted curves are seen to deviate from this continuous line right from their origin, and this deviation is attributed to the potential produced by the newborn impulses which are also set up by the second nerve volley.

lengthening of the volley interval causes the second nerve volley to set up in the normal muscle firstly newborn and then fully propagated impulses. No investigation has been made of the end-plate potentials after curarization in other experiments which showed a double plateau

There is, therefore, no experimental evidence which directly indicates the site of origin of the smooth negative potential. Nevertheless, the potentials arising at other junctional regions seem undoubtedly to be produced on the far side of this junction, and at the neuro-muscular junction we have the histologically specialized region of the motor end-plate, so that this region seems to be the only probable site for the production of potentials which have no known analogy in the responses of nerve or muscle fibres themselves.

Thus it may be concluded that, in refractory muscle, a nerve volley sets up two processes which produce a negative potential change localized to the end-plate region: one process is the "newborn" impulse which fails to grow up to a fully propagated impulse, and is produced in the muscle fibres themselves; the other process, which gives a smooth negative potential wave, occurs even when no newborn impulses are set up, and so is without parallel in muscle and nerve fibres, the specialized region of the muscle fibre forming the motor end-plate being the only probable site for its production—henceforth this potential will be called the end-plate potential.

(c) *The "newborn" muscle impulses.* In a muscle poisoned with curare so that a nerve volley fails to set up propagated muscle impulses and the accompanying contraction, this volley still sets up potential changes localized to the region of the end-plates [cf. Schaefer & Göpfert, 1937]. Further it has been shown [Eccles & O'Connor, 1938*b*, 1939*d*] that these potential changes are produced by the same two processes described above: newborn impulses which fail to grow up into fully propagated impulses; and a specialized response of the motor end-plates which gives the true end-plate potential and which is produced even when the curarization is so deep that no newborn impulses are set up. The time courses of these two potentials of the end-plate region closely resemble the corresponding potentials produced, as above described, by a nerve volley in refractory muscle.

In muscle curarized so deeply that a nerve volley only sets up an end-plate potential, a second nerve volley superimposes its own end-plate potential on this, the size of the additional potential being less than that of the first potential—usually about 80% of it—until the volley interval is several seconds. With lengthening of the volley interval from a very short value, the potential-interval curves of Fig. 19 show that the increase in the end-plate potential set up by the second volley initially runs parallel with that observed for normal muscle. However, a deviation is observed beyond a volley interval of 1.0 msec. in Fig. 19*B*, there being a much smaller second step in the end-plate potential of the curarized

than the potential curve for the abortive impulses plus end-plate potential set up by the second volley at 1.8 msec., the sudden deviation from this curve forming an inflection which presumably signals the transition from the newborn to the fully propagated muscle impulses. As the volley interval is lengthened, the first stage of the potential is seen to become still earlier and steeper, the inflection occurring correspondingly earlier and being less evident, until with a volley interval of 3.1 msec. (Fig. 13A) it is barely detectable. The fully propagated impulses must then arise less than 0.2 msec. after the beginning of the action potential, and at still longer intervals, e.g. 4 msec., probably in about 0.1 msec. On our previous assumption in regard to the end-plate potentials, the increasing steepness of the first stage of these action potentials must be due to the larger potentials produced by newborn impulses set up by the progressively later second nerve volley, and *pari-passu* these newborn impulses are observed to grow up more rapidly into fully propagated impulses.

In the eleven experiments of this type which we have performed on soleus muscles with localized end-plate leads, similar results have always been obtained (cf. Fig. 15). In some the potentials set up by the second volley could not be calculated as accurately as in Figs. 13 and 15, but in all there was the general indication that the newborn impulses, when propagated, arose at least as soon as the end-plate potential. The inflection signalling the origin of the fully propagated impulses was also regularly observed.

(d) *The effect of volley interval on the time course of the response to the second volley.* The detailed effects of variation of the volley interval on the muscle impulses set up by the second volley are well illustrated by the method of plotting adopted in Fig. 21, which shows a typical series of observations. The volley intervals are plotted as abscissae in all the six curves, and from below upwards the ordinates of the curves are: the latent period of the summit of the negative muscle spike evoked by the second volley and recorded (1) at the end-plate zone, (2) 10 mm., and (3) 25 mm. distal from this zone; the maximal potential of this negative spike recorded (4) at the end-plate zone, (5) 10 mm. distal from that zone, and (6) the maximal tension of the contraction evoked by the two nerve volleys (the contraction-interval curve of Figs. 10 and 16). From the argument already developed in relation to the contraction-interval curve, the two perpendicular broken lines drawn in Fig. 21 will show the shortest volley intervals at which the second volley sets up a contraction response (a) in any muscle fibres, (b) in all the muscle fibres. The horizontal broken

in the potential-interval curve, e.g. that of Fig. 17E. But in none of our experiments on curarized muscle has a double plateau been certainly observed. It thus appears likely that the second step in the potential interval curve is usually depressed by curare as is observed in Fig. 19B, but further investigation is required.

In curarized muscle the time course of the end-plate potential is unaltered by lengthening the volley interval, but its origin and rising phase move slightly earlier until the volley interval is about 2 msec. in duration. A similar behaviour is observed with normal muscles (cf. Figs. 13, 15) until the volley interval is so long (about 1.6 msec.) that newborn impulses are superimposed on the end-plate potential. Thus up to this interval the only difference between the end-plate potentials of normal and curarized muscles would appear to lie in the much larger second step sometimes observed in the normal potential-interval curve. The similarity thus observed when the second step is absent or insignificant (Figs. 17A, B, C), provides a justification for the assumption that this similarity of the normal and curarized end-plate potentials also obtains at longer volley intervals, i.e. when it is obscured by the newborn and fully propagated muscle impulses then set up in normal muscle. For example, on this assumption the end-plate potential observed for intervals of 1.25–1.4 msec. in Fig. 13A need only be moved a little earlier (as indicated by the curarized responses) when plotting its course at the longer volley intervals. Fig. 20 shows that at all volley intervals the potential thus attributed to the newborn impulses probably begins slightly earlier than this calculated end-plate potential. Certainly it is not later, for the combined potential curve deviates from the end-plate potential right from its origin. Even where a large second step is observed in the normal potential-interval curve the plateau following it suggests that no further increase in the end-plate potential is produced by lengthening the volley interval. It would then seem justifiable to assume that in these experiments, too, the end-plate potential at longer volley intervals may be plotted by moving slightly earlier the end-plate potential observed during the second plateau.

In some experiments—particularly those where the aborting newborn impulses give rise to a relatively small potential—the early origin of the newborn impulses cannot be demonstrated as described above. But it is always observed when the volley intervals are so long that the second volley sets up fully propagated impulses. The potential which is set up by the second volley now shows a two-stage rise. With 1.9 msec. interval in Fig. 13A, the first stage of about 0.6 msec. is but little steeper and earlier

With the spike potentials recorded 10 and 25 mm. from the end-plate zone (curves 2 and 3), a definite shortening of latent period is observed with volley intervals as long as 16 msec., and this shortening increases to a maximum as the volley interval is diminished to about 6 msec., again decreasing and finally reversing into a lengthening at about 3 msec. Comparison with curve 1 shows that a diminution in the neuro-muscular delay produces only a small part of this shortening, and then only at volley intervals less than 8 msec. The observed shortening of latent period must, therefore, be chiefly due to a supernormal conduction velocity of the muscle impulses, which is also indicated by its progressive increase with the distance of transmission, as shown by a comparison of curves 2 and 3. In Fig. 21 the normal velocity of 4.05 m./sec. is increased to 4.35 m./sec. at the height of this supernormality. A phase of supernormal conduction velocity with a similar time course has been observed in all our experiments, the longest volley intervals at which it is detectable varying from about 15 to as much as 40 msec. Since this supernormal phase of impulse conduction is also observed with direct stimulation of curarized muscle, it would appear to be purely an attribute of the propagation of impulses in the muscle fibres, and independent, as would be expected, of their mode of origin.

A more detailed study of the responses set up by the second volley at brief volley intervals may be made by reference to Fig. 22 in which are plotted the observations already illustrated in Figs. 12, 13 and 19A, as well as those in the curarized muscle, the volley intervals being the abscissae in every curve just as in Fig. 21.

The latent periods of the negative potentials are plotted as ordinates in curves 1 and 2, the former being for the curarized and the latter for the normal muscle. In general the curves are not dissimilar, but, in its greater shortening of latent period at intervals longer than 1.5 msec., the normal curve possibly differs significantly from the curare curve—particularly as it has a somewhat longer latent period at the very brief volley intervals. The approximate 45° slope for intervals up to 1.3 msec. has been a regular feature of the latent periods both of normal and of curarized responses (in Fig. 22 there are insufficient observations for the curarized muscle, but in a later paper a more complete series will be illustrated [Eccles & O'Connor, 1939*d*]). This slope of 45° indicates that at these brief intervals (0.95–1.2 msec.) the second volley is setting up the negative potential at a constant interval (about 1.45 msec. in normal and 1.35 msec. in curarized muscle) after the beginning of the negative response to the first volley.

lines show for each curve the corresponding control values for a response evoked by a single nerve volley.

As the volley interval is progressively diminished, the spike latent period at the end-plate zone (curve 1) first shows a slight shortening for volley intervals less than 8 msec. There is a reversal of this shortening

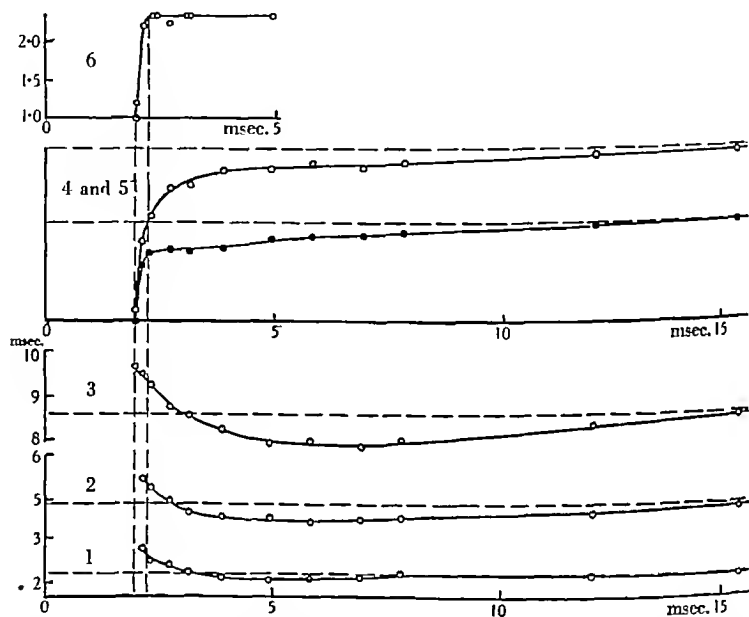


Fig. 21. The six curves show the effect of volley interval on the response to the second nerve volley. Volley intervals are plotted as abscissae, and as ordinates are plotted the following measurements of the respective responses: (1) the latent period of the summit of the negative spike at the end-plate zone; (2) 10 mm.; and (3) 25 mm. distal from this zone; (4) the maximum potential of the negative spike at the end-plate zone; (5) 25 mm. distal from this zone (solid circles); (6) the contraction interval curve. The horizontal broken lines show the corresponding control values for the single response.

to a lengthening at about 3 msec. volley interval, and a further progressive lengthening until the volley interval is so brief that the spike is extinguished. These changes have been observed in all our experiments, and suggest that at intervals from about 3 to 8 msec. a second volley sets up muscle impulses after a neuro-muscular delay shorter than normal, while at intervals less than 3 msec. this delay is longer than normal. Similar effects of volley interval on the synaptic delays of ganglia have already been described and the shortening has been called temporal facilitation [Eccles, 1935*b*].

In curve 4 the ordinates are the latent periods of the crests of the negative spikes—the most accurate of all our time measurements. The shortening of these latent periods at intervals beyond 3 msec. is again shown (cf. curve 1, Fig. 21), and it is now possible to see in detail the effect of diminishing the volley interval below 3 msec. There is then a progressive lengthening of the latent period beyond its normal value, and, for volley intervals from 2.1 to 2.3 msec. the curve rises at about 45° , indicating that there is a constant interval (2.5 msec.) between the negative crests set up by the two volleys. Nevertheless, at still briefer volley intervals, the curve becomes steeper than 45° , indicating that the negative crest interval has lengthened. The observation at 1.9 msec. showing a lengthening of 0.1 msec., i.e. the crest interval, is 2.6 msec. This lengthening, though small, is well within the limits of our accuracy of measurement (about 0.02 msec.) for these crest times, and has been observed in all of our eleven experiments in which a sufficient series of observations were made at brief volley intervals. In one experiment the maximum lengthening was as much as 0.3 msec., but usually it ranged about 0.1 msec. Though plotted differently, curve 4 of Fig. 22 is an example of the *C* type of curve which Lucas [1910] obtained for the sciatic-gastrocnemius preparation of the frog. His observations, however, showed that with very brief volley intervals there is a much greater lengthening of the muscle response interval than we have observed, and Lucas may actually have been observing the delayed muscle responses which we are describing in a later paper.

In curve 3 of Fig. 22 the ordinates are the latent periods of the inflections which are presumed to represent the origin of the "fully grown" muscle impulses. In general features this curve is similar to curve 4, showing that a slight shortening of latent period changes to a lengthening as the volley interval is diminished below about 3 msec. Again, there is with brief volley intervals the rise of the curve at about 45° , but this does not merge into a still steeper rise as in curve 4. The interval between the origins of the impulses set up by the first and second volley remains constant at about 2.35 msec. Possibly this difference between curves 3 and 4, and also the smaller shortening of latent period at intervals of 3–4 msec., is attributable to the former curve plotting changes in the minimum latent periods, i.e. in the minimum neuro-muscular delays, and the latter curve changes approximately in the modal class of the neuro-muscular delays. A similar difference has been observed for the synaptic delays in transmission through sympathetic ganglia, the longer synaptic delays being diminished by

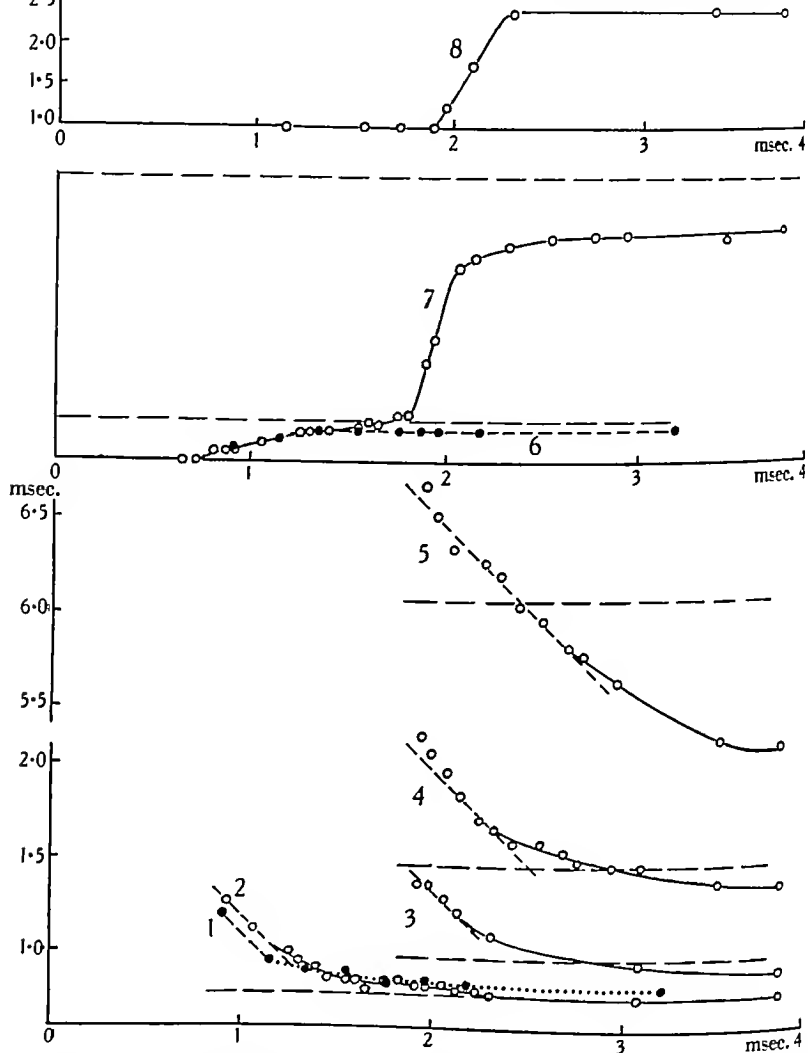


Fig. 22. A series of observations from another experiment plotted in general as in Fig. 21, but only short volley intervals are shown. As ordinates are plotted the following measurements of the respective responses: (1) the latent periods of the beginnings of the negative responses in the curarized muscle (solid circles and dotted line); and (2) in normal muscle (open circles); (3) the latent periods of the inflections signalling the origin of the fully grown impulses at end-plate region; (4) the latent periods of the summits of the muscle spikes at the end-plate region; and (5) 22 mm. distal from this region; (6) the maximum negative potential set up at the end-plate zone of the curarized muscle (solid circles and broken line); and (7) in normal muscle (open circles). The earlier parts of curves 6 and 7 have already been shown in Fig. 19A. Curve 8 shows the contraction interval curve obtained a little later in this experiment. Comparison with curve 7 indicates that the critical intervals have been a little lengthened. In curves 1, 2, 3, 4 and 5 broken lines are drawn at 45° in relation to the observations at the shortest volley intervals. The horizontal broken lines show for each of the curves 2, 3, 4, 5, 6 and 7 the corresponding control measurements of the response set up by a single nerve volley. The line for curve 1 is not drawn, but it passed through the latter part of the dotted line.

the contraction-interval curve (curve 6 in Fig. 21) is due to a progressive increase in the number of muscle fibres responding to the second volley. Correspondingly, for this range of volley intervals, there is a rapid increase in the spike action potential set up by the second volley (curves 4 and 5 in Fig. 21). This increase, however, is only to about two-thirds of the spike potential set up by a control single volley, and further lengthening of the volley interval to 16 msec. is accompanied by a gradual increase in the action potential to approximately its full control value. Our experiments on the contraction (Fig. 11) show that this gradual increase is not due to the response of additional muscle fibres. A similar delayed increase was observed in all our experiments on soleus, but some three of these experiments differed from Fig. 21, for, with volley intervals longer than 8 msec., the phase of subnormal action potentials passed over into a phase of slightly supernormal potentials, particularly with those potentials recorded some distance away from the end-plate zone.

The subnormal action potentials must be due either to decreased action potentials of individual muscle fibres, or to an increase in their temporal dispersion, whereby, by increase in their mutual interference, normally sized action potentials of individual fibres seem to give a smaller aggregate action potential. This latter factor could not play a part with intervals longer than about 3.0 msec., for then the neuro-muscular delays and the conduction times of the muscle impulses are shorter than normal, and hence there would be a diminished temporal dispersion. This diminution in temporal dispersion is probably responsible for the late supernormal phase occasionally seen. The subnormal phase, particularly at the longer volley intervals, must be due to a diminution in the size of the individual muscle impulses (see section E 3).

The potentials produced at the end-plate region by a second volley at brief volley intervals have already been discussed (sections B 2 (b) and (c)) and illustrated by Figs. 12, 13 and 15, and are also shown in the early parts of curves 6 and 7 in Fig. 22, in which are plotted the curves of Fig. 19A on a different scale. As shown in Fig. 17, lengthening of the stimulus interval beyond a critical value, varying in different experiments from 0.65 to 0.8 msec., results in the production of an end-plate potential by the second stimulus. This end-plate potential may reach a plateau when the stimulus interval is as short as 0.7 msec. (Fig. 19B), or only when it is as long as 1.25 msec. (Fig. 19B). In these two extreme experiments a similar divergence was observed for the production of an end-plate potential in curarized muscle.

temporal facilitation to a much greater extent than the shorter [Eccles, 1935b].

In curve 5 of Fig. 22 the ordinates are the latent periods of the spikes recorded by the electrodes distal to the end-plates as the muscle impulses reach this electrode after transmission for 2.2 cm. along the muscle fibres (cf. curves 2, 3, Fig. 21). Comparison with curve 4 shows at the longer volley intervals the large additional shortening of latent periods arising on account of the supernormal conduction velocity of the muscle impulses. The normal velocity of 4.8 m./sec. is increased to as much as 5.5 m./sec. At the briefer volley intervals the curve rises at 45° , indicating that at these distal muscle electrodes the response interval of 2.4 msec. is independent of the nerve volley interval so long as this is less than 2.7 msec. This minimum response interval recorded at the distal electrodes (2.4 msec.) is thus actually slightly shorter than the minimum response interval for the negative crests recorded at the end-plates (2.5 msec.). Thus even at this brief interval of 2.5 msec., there must be a slight supernormality of conduction rate. With larger stimulus intervals the supernormality is much greater, hence during its propagation along the muscle fibre the second impulse tends to catch up to the first impulse until it gets within about 2.4 msec. Thus there is a 45° rise in curve 5 for a large range of volley intervals. This range would, of course, be still larger if a longer muscle conduction path were available.

Supernormality of conduction is not usual for such brief intervals as in Fig. 22. For example, in Fig. 21, 2.8 msec. is the minimum interval at which supernormality occurs. Here impulses set up at a briefer interval than 2.8 msec. are conducted with a subnormal velocity until they are at an interval of 2.8 msec. In most of our experiments the second nerve volleys at very brief intervals (less than 3 msec.) have set up muscle impulses conducted with a subnormal velocity, but in three experiments the velocity of these earliest muscle impulses did not differ significantly from normal, i.e. they were neither supernormal or subnormal, while in the experiment illustrated above these earliest impulses were actually conducted with a slightly supernormal velocity. Reference may be made to Forbes, Ray & Griffith [1923, p. 579] for an account of the effect of conduction distance on the lagging produced by subnormally conducted impulses. There it is shown that only after conduction for a considerable distance does this lagging give rise to a constant interval between the first and second impulses.

(e) *The effect of volley interval on the size of the action potentials produced by the second volley.* It has been shown that the rising phase of

experiment. With 1-2 stimulus intervals shorter than 0.8 msec., the second stimulus gives no significant end-plate potential, but the third stimulus (0.4 msec. after the second) gives the full plateau height of this potential, the response to stimuli 1, 2 and 3 being practically identical with that to stimuli 1 and 3. The second stimulus at intervals shorter than 0.8 msec. therefore excites at most an insignificant number of nerve fibres, its failure to produce an end-plate potential being entirely due to its failure to set up impulses in the motor nerve fibres. With 1-2 intervals of about 0.82 msec., the second stimulus evokes a small end-plate potential, and this is increased to plateau height by a third stimulus. 0.4 msec. later. Again, one must conclude that the second stimulus sets

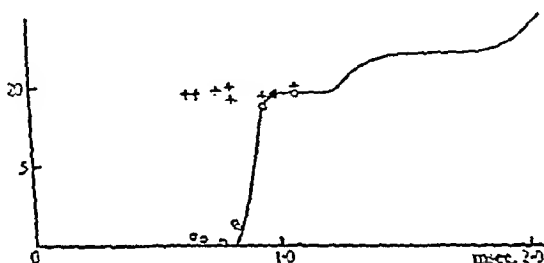


Fig. 23. A potential interval curve is drawn from the series of points shown in Fig. 17 E. The open circles are a further series of these points, i.e. the maximum negative potential added by the second nerve stimulus is plotted against the stimulus interval. The crosses show the maximum negative potential added to the response to the first stimulus when a third stimulus is applied 0.4 msec. after the second, i.e. by the second and third stimuli combined, the abscissae still being the interval between the first and second stimuli. Further explanation in text.

up a small end-plate potential because it excites only a few motor nerve fibres. With a slightly longer stimulus interval (0.95 msec.), the second stimulus evokes an almost full-sized end-plate potential, the third stimulus adding only a small amount to bring it to the plateau height. Thus this experiment shows that explanation 1 is correct, explanations 2, 3 and 4 at the most being insignificant in determining the increase of end-plate potential to plateau height as the stimulus interval is lengthened. Similar observations have been made in six other experiments, but no conclusive evidence has been obtained that explanations 2, 3 or 4 play a significant part in the first step of the potential interval curve.

Therefore as soon as the motor nerve fibres have recovered from their absolutely refractory state, impulses can be propagated along their profusely branching peripheral course to the terminal endings in the motor end-plates, which are thus excited to give an end-plate potential.

The following four explanations may be offered for this initial rising phase of the potential-interval curve.

(1) There is a temporal distribution of the absolutely refractory periods of the individual motor nerve fibres, the rising phase of the end-plate potential being due consequently to the second stimulus setting up impulses in more and more motor fibres until eventually all respond as the plateau is reached.

(2) The second stimulus sets up impulses in the motor nerve fibres at shorter intervals than the critical intervals shown in Figs. 18 and 19, but these impulses are blocked on the way to the muscle on account of the progressive lengthening of the absolutely refractory periods which would be associated with the diminution in size of the motor nerve fibres at each of the many bifurcations which they undergo on their short peripheral course to the motor end-plates.

(3) The second stimulus sets up impulses as in (2) at intervals shorter than the critical intervals, and these impulses actually reach the motor end-plates, but are so small on account of the relative refractoriness of the nerve fibres that the end-plates are inadequately excited, consequently giving either no potential response, or a potential response less than that set up by a full-sized nerve impulse.

(4) The end-plates themselves may exhibit a refractoriness after the response to the first nerve volley, the second nerve volley setting up no response or a diminished response until the end-plates have recovered.

When the stimulus interval is such that the second stimulus either partly or completely fails to set up an end-plate potential, then, according to the first explanation, this is entirely due to its failure to set up impulses in the motor nerve fibres, while according to explanations 2, 3 and 4 it sets up impulses, but these fail for various reasons in setting up an end-plate potential. Thus, according to explanation 1, the second stimulus sets up impulses and a subsequent refractory period only in those motor nerve fibres whose end-plates give a full potential response, while, according to explanations 2, 3 and 4, the second stimulus should set up impulses and a subsequent refractory period in other motor nerve fibres as well.

The presence or absence of a refractory period after the second stimulus may be tested by applying a third stimulus at not more than 0.4 msec. after the second. Such a stimulus will fall during the absolutely refractory period of any fibres that respond to the second stimulus; hence nerve fibres may respond either to the second stimulus or to the third stimulus, but not to both. Fig. 23 shows the results of such an

picked up to a significant extent by either electrode; hence the localized action potentials so recorded would form a much smaller fraction of the muscle spike potential than with our direct leads from localized groups of end-plates. Our failure to detect such potentials in all but a few doubtful experiments on intact muscle is, therefore, not surprising. However, when the muscle spike potentials are abolished by curarization of the muscle, the typical end-plate potentials are always observed if one recording electrode is placed on any part of the muscle in which there are end-plates. Even in the detailed measurements the figures for the intact muscle are practically identical with those for the localized strip; e.g. the shortest interval at which a second impulse can be set up in the muscle is 2.2-2.6 msec. for our intact muscle preparations, and 2.45-2.8 msec. for our localized preparations. Hence it may be concluded that the localized group of end-plates and their muscle fibres investigated in our experiments are a fair sample of the soleus muscle, our measurements on them being applicable to all other fibres of soleus muscle.

C. THE ACTION OF TWO NERVE VOLLEYS ON THE MUSCLES TIBIALIS ANTICUS AND PERONAEUS TERTIUS

In the essential features two nerve volleys have been shown to set up responses in tibialis anticus and in peroneus tertius (see Fig. 9) which closely resemble those described in section B for soleus muscles, the only significant differences arising on account of the quicker time course of some responses of tibialis anticus and peroneus tertius. This quicker time course has made our observations on the end-plate potentials and the newborn impulses less accurate than with soleus but, nevertheless, the same general features could be recognized. For example, the end-plate potential and the potential due to newborn impulses which died out before growing into fully propagated impulses were distinguishable.

Table I gives the averages of our measurements for the three muscles, those for peroneus tertius being drawn from our five experiments with localized end-plates, and those for tibialis anticus from our one successful experiment with localized end-plates and (where possible) from our six experiments on the intact muscle which were uncomplicated by repetitive muscle discharges [cf. Eccles & O'Connor, 1938a].

This table shows that the tibialis anticus and peroneus tertius muscles are very similar in all respects where a comparison has been possible, and are both somewhat quicker in most of their reactions than soleus. This difference is still more marked in the contractions of the respective muscles. Peroneus tertius is a quick muscle like tibialis

This does not imply that the absolutely refractory periods of the fine terminal motor fibres are as short as those of the large fibres at the stimulated region of the nerve. The impulses set up there at the end of the absolutely refractory period will lag considerably during their short peripheral pathway—perhaps a slight delay occurs at each bifurcation of the nerve. Clark's counts [1931] show that on the average there are about seven bifurcations in the peripheral pathway of the motor nerve fibres to soleus, and these will be almost entirely in the motor nerve fibres peripheral to our stimulation point [Eccles & Sherrington, 1930].

The values shown in Fig. 17 for the shortest absolutely refractory periods of the soleus motor nerve fibres at the point of stimulation thus vary from about 0.65 to 0.80 msec. These values are considerably longer than those (0.4–0.5 msec.) found by Gasser & Grundfest [1936], and by Graham & Lorente de N6 [1938] for the fastest mammalian nerve fibres. However, as pointed out in section B1, it must be remembered that soleus nerve fibres are considerably smaller in diameter than the largest mammalian nerve fibres; hence a longer refractory period would be expected. In all experiments but one of those illustrated in Fig. 17 the longest absolutely refractory periods of the soleus motor nerve fibres were indicated to be little if any longer than 1 msec., being as short as 0.7 msec. in one experiment. In the exceptional experiment (cf. Fig. 19A) the upper limit would have to be as long as 1.25 msec., which seems unaccountably long for motor nerve fibres. The temperature of both the cat and the box was 38° C., and the stimuli were rather more than seven times the just maximal strength. A possible explanation of such a prolonged absolutely refractory period is that some of the motor nerve fibres had already subdivided considerably by the time they had reached the stimulating electrodes. Unfortunately, a series of observations, using a third stimulus as in Fig. 23, was not carried out in this experiment, so it may be that one of the above explanations 2, 3 or 4 was responsible for this delayed rise to the plateau. Further investigation of such slowly rising potential-interval curves is necessary. The second step of potential-interval curves such as those of Figs. 17D and E will be considered in section E2.

(f) *Observations on intact soleus muscles.* With the exception of the action potentials localized at the end-plate region, all of these observations on soleus muscles prepared with localized end-plates were originally made in our experiments on intact soleus muscles. With any position of the recording electrodes on the intact muscle, the action potentials produced at only a small fraction of the end-plates would be close enough to be

series of single maximal contractions. During a series of double responses the height of the single contraction rapidly equilibrates at its potential level, and on cessation of the series this potentiation rapidly declines to reach a new equilibration position in a few minutes. Potentiation of a single maximal contraction may also be observed shortly after a previous single or double contraction. Testing at various intervals shows that this potentiation declines rapidly and becomes undetectable in about 1 min. The action potentials show no change during potentiation except a slight forward movement of the diphasic trough indicating a faster conduction of the muscle impulses. There is no evidence that the potentiation is due to a response of additional muscle fibres or to a repetitive response of muscle fibres. It would, therefore, appear to be a true staircase phenomenon and due to an increase in the contraction of the individual muscle fibres, being thus a similar phenomenon to the post-tetanic potentiation recently described by Brown & Euler [1938]. A different type of potentiation will be described in a later paper and shown to be due to a repetitive response of muscle fibres to a single nerve volley [cf. Feng, Lee, Meng & Wang, 1938].

E. DISCUSSION

(1) *The absence of a repetitive muscle response to a single nerve volley*

In the experiments under consideration in this present paper a single nerve volley has set up the discharge of no more than a single muscle impulse from any motor end-plate. Those experiments characterized by a repetitive response of end-plates [cf. Eccles & O'Connor, 1938*a*] will be considered in a later paper.

When the muscle is prepared with a localized group of end-plates, the spike action potential set up by the discharge of the initial impulse from each end-plate is so sharp that the action potentials produced by any repetitive discharges give rise to later discrete spikes which are easily recognizable. An occasional source of confusion may occur if the preparation has been incomplete, there being two or more localized groups of end-plates in the muscle. Shift of the recording electrodes to various parts of the muscle shows that, under such conditions, the discharge of a single muscle impulse from each end-plate gives a repetitive spike response only with certain positions of these electrodes; hence this repetitive action potential is easily distinguishable from that produced by a repetitive discharge of impulses from the end-plates, for the repetitive nature of this action potential is unaffected by change in electrode position.

TABLE I

	Soleus	Tibialis anticus	Peroneus tertius
Upper limit for minimum neuro-muscular delay (msec.)	0.6	0.65	0.6
Spike potential (crest time) (msec.)	0.75	0.6	0.6
Spike potential (total duration) (msec.)	2.2	1.6	1.6
Muscle conduction velocity (modal class) in m./sec.	3.4	6 (only one observation)	5.0
Least nerve volley interval for adding end-plate potential (msec.)	0.8	—	0.7
Least nerve volley interval for newborn impulse (msec.)	1.6	—	1.0
Least nerve volley interval for fully-grown muscle impulse (msec.)	1.9	1.1	1.2
Least nerve volley interval for maximal muscle response to second volley (msec.)	2.3	1.8	1.7
Least muscle response interval (msec.)	2.5	2.2	1.9
Latent period of crest of end-plate potential (msec.)	2.0	—	1.7
Time to half decay of end-plate potential (msec.)	5.0	—	4.0

anticus, the contraction time of their twitches being about 30 msec., while the contraction time of a soleus twitch is as long as 100 msec. [cf. Cooper & Eccles, 1930].

The qualitative similarity of the responses of these different muscles to one or two nerve volleys indicates that the essential features in the transmission from nerve to muscle are identical, the quantitative variations in time course presumably being dependent on intrinsic differences in the time constants of the muscle fibres themselves and of their specialized parts.

In the discussion later in this paper consideration will be restricted to neuro-muscular transmission in soleus muscle whose slower time constants allow a more accurate investigation; but there is every indication that the conclusions derived therefrom also hold for tibialis anticus and peroneus tertius, and hence presumably for other mammalian striated muscles.

D. THE STAIRCASE PHENOMENON

In several of our experiments the contraction evoked by a single nerve volley has shown a progressive increase, even though there has been an interval of 30 sec. between successive observations. In some experiments on tibialis anticus it has been as large as 20%, in others it was barely detectable. The largest increases follow the large contractions evoked by double nerve volleys and then appear to be independent of the volley interval so long as this is long enough to set up a double response of the muscle. A smaller progressive increase is also observed during a

(3) If the contraction interval curve shows the steep symmetrical S-shaped rise (cf. Figs. 10, 16) to a plateau with a later gradual fall, there must be a symmetrical distribution of the muscle fibres in so far as their response to one or two nerve volleys is concerned. There is no tendency for a special grouping as would be expected if some muscle fibres responded repetitively to a single nerve volley. The complications produced in the contraction-interval curve by such repetitive responses will be described in a later paper.

The above lines of evidence will be better illustrated in the later paper on muscle responses which will be classed as repetitive on all the above tests. For the experiments described in this present paper it may be concluded that there is no evidence that a repetitive muscle response is set up by a single nerve volley. The term "muscle spike" may be applied to the action potential which is produced when a single nerve volley sets up the discharge of no more than a single impulse in each muscle fibre.

(2) *The end-plate potential*

With normal soleus muscles the pure end-plate potential only is set up by a nerve volley which is less than about 1.6 msec. after a previous volley. Its close similarity to the end-plate potential produced by a second volley in deeply curarized muscle (both in time course and in the early part of the potential-interval curve, cf. Fig. 19) suggests that at longer volley intervals the end-plate potentials in normal and in curarized muscles are also similar. It further suggests that in normal, as in curarized muscle, the *first* nerve volley also produces a true end-plate potential which is similarly related to the potential set up by the second volley at intervals less than 1.6 msec. The existence of such a potential in normal muscle is substantiated by the action of subparalytic doses of curare [cf. Eccles & O'Connor, 1939*d*]. Under such conditions there is a small diminution in the negative potential recorded with an electrode on the end-plate zone. Subtraction from the normal action potential set up there by a single nerve volley shows that this diminution has a similar time course to the end-plate potential. Thus it may be concluded that a single nerve volley sets up an end-plate potential in normal muscle, and that this potential is diminished by subparalytic doses of curare just as the pure end-plate potential of a completely curarized muscle is further diminished by additional curare [Eccles & O'Connor, 1938*b*]. Further evidence for the production of an end-plate potential in normal muscle by a single nerve volley is provided by the action of subrepetitive doses of eserine in causing a late increase in the negative potential set up at the end-plate

diminishing relative refractoriness during which a subnormal response is evoked by a nerve impulse, the rapid recovery from this period giving the second step of the potential-interval curve.

This explanation is based on an analogy with the all-or-nothing propagated impulses in nerve and muscle fibres; hence it seems most improbable on general grounds, for there is no evidence that the end-plate potential even remotely resembles such an impulse. Thus it is possible to distinguish sharply between newborn impulses at the end-plate region and the true end-plate potentials under consideration. Moreover, in the observed summation of end-plate potentials, there is no indication of an all-or-nothing character of the end-plate potential [cf. Adrian, 1933], for a second potential beginning 1.4 msec. after the first will arise even before the summit of the first potential (cf. Fig. 25B), and will often be just as large as the end-plate potential arising late on the decline of a previous end-plate potential, or even several seconds after this decline.

(2) Alternatively the motor end-plate has no true refractory period in its response to nerve excitation. The increased delay of the end-plate potential with short stimulus intervals would be entirely due to delay in the excitatory action of the second nerve impulse, and the size of the end-plate potential would, in addition, exhibit some gradation according to the size of this nerve impulse.

On this explanation the interval between the arrival at the motor end-plates of the two nerve volleys would be constant at about 1.35 msec. for the four points on the first plateau of Fig. 17D, the second nerve volley consequently having a constant size; hence the constancy of the response of the end-plates both in its size and in its time course. With the observations of the second step the further lengthening of the stimulus interval would be associated with an increasing interval between the bombardment of the motor end-plates by the two volleys, the second nerve volley thus being progressively larger in size on account of the more complete recovery of the nerve fibres from their refractory period; hence it evokes a progressively larger end-plate potential. With still further lengthening of the stimulus interval, the second nerve volley approaches its full size and evokes the almost constant end-plate potential of the second plateau. Thus, in Fig. 17D, a second stimulus 0.7 msec. after the first, would have to evoke a nerve volley bombarding the end-plates 1.35 msec. after the first. i.e. the conduction time would be 0.65 msec. longer than normal. Such a delay would probably be necessitated by the lengthening of refractory period in the fine terminal branches of the motor nerve fibres, for these would be expected to have an

zone by a single nerve volley. This increase closely resembles the increase produced by eserine in the pure end-plate potential of completely curarized muscle [Eccles & O'Connor, 1939c].

It was shown in section B 2 (e) that nerve impulses set up just at the end of the nerve's absolutely refractory period traversed the fine branching peripheral nerve pathway and excited the motor end-plates to produce an end-plate potential. In some experiments on normal muscles and in most experiments on curarized muscles this end-plate potential was practically as large as that produced by nerve impulses set up much later (up to several seconds in curarized muscle) after the refractory period. However, the potential-interval curves of some experiments show, in addition, a second step-like increase in the end-plate potential to a second plateau (Figs. 17D, E), this step beginning with volley intervals of 1.1–1.3 msec. Its separation from the first step by a definite plateau indicates that this second step is separately produced, and not due to the recovery of an additional group of nerve fibres from absolute refractoriness either at the point of stimulation or in their profusely branching peripheral course. Thus this second step would be due to an increase in the end-plate potential of the individual motor end-plates.

This inference is supported by an examination of the latent periods of the end-plate potentials before, during and after this second step. With very short volley intervals the latent period of the end-plate potential set up by the second volley is an inverse function of the volley interval (cf. curves 1 and 2, Fig. 22). For example, in Fig. 17D, the second end-plate potential is set up at a practically constant interval (1.35 msec.) after the first during the four observations of the first plateau. During the rising phase of the second step, this response interval lengthens and again further increases from about 1.5 to 1.65 msec. during the slightly rising second plateau. It appears that the second step is associated with the early stages of the increase in the interval between the successive responses of the end-plates. This association suggests the following alternative possibilities.

(1) After the response of a motor end-plate to bombardment by a nerve impulse there is a period of about 1.4 msec. during which a second nerve impulse cannot set up an additional end-plate potential. The excitatory action of such a nerve impulse is delayed and sets up an end-plate potential at the end of this absolutely refractory period of the end-plate, the first plateau of the potential-interval curve showing that the size of this response is independent of the delay. Following on this absolutely refractory period there may be a short period of rapidly

set up by this volley. The calculated end-plate potential is observed to begin slightly later than the total potential and to rise at least four times more slowly right from the start. An essentially similar relationship has been observed in every one of our experiments in which the end-plate potential could be calculated. Presumably the large additional potential is due to the newborn impulses, which are thus clearly shown to be set up at least as soon as the end-plate potential. The spike potential set up by a single nerve volley in normal muscle always shows a relatively gradual onset with a much steeper rise beginning about 0.15-0.2 msec. later. Presumably the newborn impulse takes this short time to flare up to the fully propagated size.

The independent production of the end-plate potential and the newborn impulse potential by the nerve impulses does not necessarily mean that two independent neuro-muscular transmitters are involved. The same transmitter could be the means of exciting both the end-plate giving the end-plate potential and the muscle fibre adjacent thereto (either directly or indirectly through the end-plate) setting up the newborn impulse potential, which, if sufficiently large, grows into the spike potential of the fully propagated impulse.

With shortening of the nerve volley interval below 2 msec., the newborn impulse potential shows only a slight lengthening of latent period until it becomes so small relative to the end-plate potential that its beginning cannot be separately distinguished. With a further slight shortening of the nerve volley interval the second volley fails altogether to set up newborn impulses. The excitatory effect whereby the second nerve volley sets up the newborn muscle impulses must, therefore, be of very brief duration; for otherwise the second volley would be effective at still shorter volley intervals, its persistent excitatory effect acting on the muscle as soon as this had recovered sufficiently from the refractory period following the first response. We have seen that in fully recovered muscle a nerve volley sets up newborn impulses with a minimum neuro-muscular delay of not more than 0.6 msec., the maximum delay being little if any more than 1.0 msec. (section A3). The maximum duration of the neuro-muscular delay at which a second nerve volley is observed to set up newborn impulses in refractory muscle is probably no more than about 1.0 msec. when allowance is made for the increased nerve conduction time of such an early second volley. Thus the excitatory action whereby nerve impulses at the neuro-muscular junction excite the production of newborn muscle impulses, cannot be demonstrated to persist for more than about 1.0 msec.

absolutely refractory period considerably longer than that of the parent fibres [Erlanger & Gasser, 1937, p. 48]. The delay possibly occurs at the points of subdivision, the maximum delay being less than 0.1 msec. per subdivision, a not improbable figure.

On both general grounds and present evidence this second possibility seems to be much more probable than the first, and it will receive further support in later papers dealing with the action of drugs such as curare and eserine.

(3) *The setting up of newborn muscle impulses*

The end-plate potential must arise independently of the newborn impulse potential, for it alone is set up in completely curarized muscle and in normal muscle by a nerve volley at a very short interval (less than about 1.6 msec. in soleus) after a preceding volley. The newborn impulse potential is never thus observed in isolation; hence the possibility must be considered that the newborn impulses are set up by the end-plate potential, being thus only secondarily produced by the nerve impulses. However, such a suggestion is rendered untenable by careful time measurements, for it has been shown in section B2 (c) that the newborn impulse potential may begin, if anything, slightly earlier than the end-plate potential. Within the limits of error it seems that, in some of our experiments at least, this temporal relationship of the newborn impulse potential and the end-plate potential obtains for all volley intervals. However, in other experiments this relationship can only be certainly demonstrated when the nerve volley interval is so long that the newborn impulse is large and rapidly grows up to a fully propagated impulse.

In section B2 (c) evidence was given suggesting that, except in experiments showing a double step in the potential-interval curve, the end-plate potential set up by a second nerve volley in normal muscle was affected by volley interval exactly as in curarized muscle. If that is so, then it would be expected that in normal and curarized muscle a similar relationship would obtain between the end-plate potentials set up by the first and second nerve volleys. On this basis the end-plate potential set up by a single nerve volley in a normal muscle has been drawn in Fig. 24 on the same scale as the beginning of the total potential

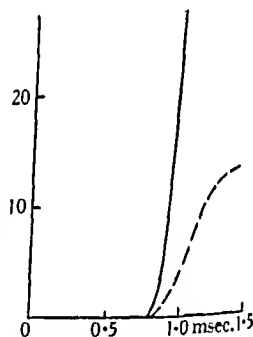


Fig. 24. The continuous line shows the early course of the potential set up by a single nerve volley, and the broken line shows the curve for the true end-plate potential set up by a single nerve volley and calculated as described in the text. Plotting as in Fig. 13.

after a delay even shorter than the normal period of about 0.2 msec. The effect of volley interval on this temporal facilitation (cf. curve 1, Fig. 21) indicates that the end-plate potential produced by the first volley is responsible for this effect. Shortly after the first response, the muscle's refractory period counteracts any such facilitating effect, and as soon as this refractory period has passed off, the brief phase (about 3-8 msec.) of temporal facilitation ensues. When curare blocks neuro-muscular transmission, the end-plate potential set up by one nerve volley exerts a facilitating effect on a second volley [Eccles & O'Connor, 1938*b*], which may thus be enabled to set up propagated impulses and a consequent contraction of the muscle [cf. Adrian & Lucas, 1912; Bremer, 1927; Katz, 1939].

The supernormal conduction of a second muscle volley from 3 to 16 or more msec. after a preceding volley presumably is analogous to the supernormal conduction of a second nerve volley in frog's nerve [Graham, 1934]. There is no corresponding process in mammalian nerve [Graham & Lorente de N6, 1938]. As such it would be expected to be associated with a negative after-potential of the muscle fibres, but with our methods of leading from the muscle no direct evidence of this potential would be obtained, though it may have been this potential that was recorded by Bishop & Gilson [1927] and by Schaefer [1936] in the frog's sartorius. Such a negative after-potential, which would be set up by a muscle impulse along the whole length of a muscle fibre, must be clearly distinguished from the true end-plate potential which is produced independently of muscle impulses and only at the end-plates. It was argued in section B2 (e) that the subnormal spike potential produced by a second nerve volley at intervals up to 16 msec. or more must be due to a diminution in the size of the individual muscle impulses along the whole length of the muscle fibres. The time courses of this subnormal spike potential and of the supernormal phase of the conduction velocity are closely parallel (cf. curves 2 and 3 with 5, Fig. 21), the subnormal spike potential being thus also possibly conditioned by a negative after-potential of the muscle fibres. In one experiment (Fig. 22) the earliest muscle impulses that could be set up by a second nerve volley were conducted with a slightly supernormal velocity, and in three other experiments the usual early phase of subnormal conduction was not detectable (cf. section B2 (d)). It must not, however, be assumed that the excitability of the muscle fibres had recovered to normal or was even supernormal before the second nerve volley could evoke a discharge in them; for in nerve the excitability recovers more slowly than the conduction velocity [Graham & Lorente de N6, 1938].

There may, however, be a considerable delay before these newborn impulses flare up into fully propagated impulses, e.g. a delay of 0.6 msec. is shown in Fig. 13A for the response at 1.9 msec. interval, and in some experiments delays of almost 1.0 msec. have been observed. This long survival of newborn impulses and their eventual growth to fully propagated impulses is aided by two factors: the progressive recovery of the muscle from its refractory state [cf. Hodgkin, 1938]; and the end-plate potential which should help in the growth of the newborn impulse in the adjacent region of the muscle fibre. During this phase of survival of the newborn impulse the end-plate potential is progressively increasing and so should exert a considerable influence in conditioning the growth of this impulse to the critical size at which it flares up to become an all-or-nothing propagated impulse. It will be seen in a later paper that the end-plate potential sometimes exerts such a large excitatory effect that it alone sets up the discharge of muscle impulses from the end-plate zone. Nevertheless, the long latent period of this discharge clearly distinguishes it from that due to newborn impulses set up directly by nerve impulses and merely helped to grow into the fully propagated size by the end-plate potential. Thus, at the shortest interval at which a second nerve volley sets up fully propagated muscle impulses, there is, owing to the above two factors, a relatively prolonged period of indecision (always more than 0.5 msec. in soleus) before the newborn impulse flares up to the fully propagated size. With longer volley intervals this period of indecision is much shorter, for, on account of the more complete recovery of the muscle, much larger newborn impulses are initially set up by the nerve impulses; and because of their size they will flare up to fully propagated impulses before the muscle has recovered as completely as in the previous instance. The fully propagated impulses will actually be then produced at a shorter interval after the first muscle response, and so at an earlier stage of recovery of the muscle's refractory period. Hence arises that small part of curve 4, Fig. 22, which we observed to be steeper than 45° —the Lucas C curve (section B2 (d)).

At longer volley intervals, e.g. 2.4–3 msec. in Fig. 22, curves 3 and 4, the still larger newborn impulses (shown by the still steeper initial phase of the action potential recorded from the end-plate region (cf. Figs. 13A, 15)) will flare up even more quickly to fully propagated impulses, but on account of their later beginning these fully propagated impulses will be separated by a longer interval from the previous muscle response. At still longer volley intervals, e.g. longer than 3 msec. in Figs. 13A and 15, the newborn impulses appear to flare up into fully propagated impulses

0.2 msec. (cf. section E3), flares up to the fully grown muscle impulse propagated at a uniform speed, as shown by the straight line, of about 3.5 m./sec. along the muscle fibre. Since 0.4 msec. probably represents the extreme range of normal neuro-muscular delays (cf. section A4), E_2M_1 will represent the earliest and E_4M_2 the latest discharge of an impulse set up by N_1E_1 . The time course of the end-plate potential is also shown in Fig. 25A to begin at E_3 slightly after E_2 (cf. Fig. 24),

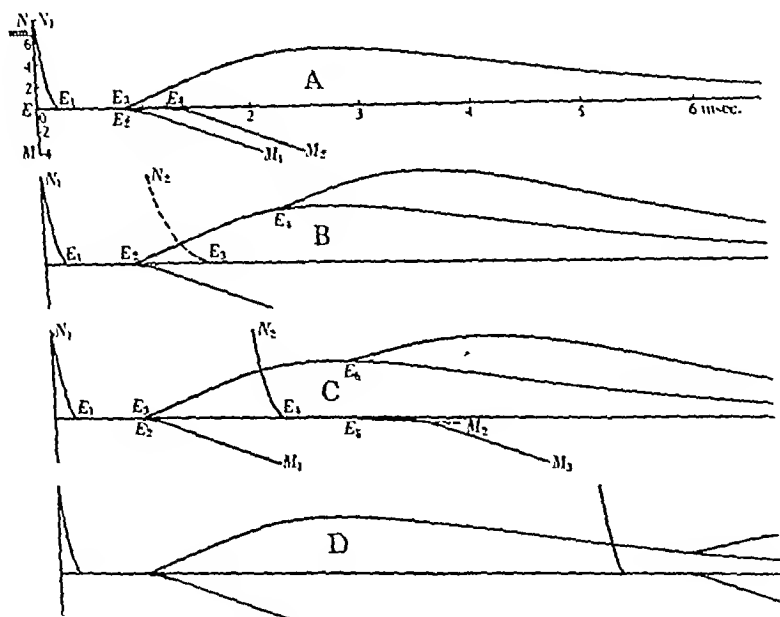


Fig. 25. Full explanation in text. (See Conclusions.)

reaching a maximum in 2-3 msec. and then decaying to half in 5 msec. This curve should really have been plotted with its ordinates perpendicular to the plane of the paper.

In Fig. 25B a second nerve volley is set up at N_2 , 1.0 msec. after N_1 , and the probable time course of this volley, the broken line N_2E_3 , is shown to be much slower than N_1E_1 (cf. section E3). No muscle impulses are set up by this volley, but the end-plate potential is shown to begin at E_4 after a neuro-muscular delay E_3E_4 not significantly differing from E_1E_2 . The two end-plate potentials are superimposed to give a considerably greater potential than that set up by the first nerve volley alone, though on analogy with curarized muscle (cf. section B2 (c)) the

Nevertheless such observations warn against a too simple interpretation of the events concerned in neuro-muscular transmission; e.g. that the nerve impulse provides a stimulus which acts directly on the muscle fibres. We shall see in later papers that the motor end-plates appear to act as a specialized intermediary station in all transmission between nerve and muscle.

Buchthal & Lindhard [1937] have recorded quick potential changes (total duration 2–3 msec.) from the end-plate region of single muscle fibre preparations in the frog and lizard. These potentials would seem to be much too rapid to be identifiable with the true end-plate potentials here described, and we have obtained no evidence suggesting that such quick potential changes are in addition produced by the end-plates. It seems possible that with their method of recording, Buchthal & Lindhard were actually observing the potential difference produced at their two closely placed recording electrodes (0.2–0.4 mm. apart) by the propagated muscle impulses. This suggestion is supported by their finding that a similar quick potential change is recorded when both leads are on the muscle fibre away from the end-plate region. The true end-plate potentials have previously been described and well illustrated by Schaefer & Göpfert [1937] in curarized frog's muscles. They also illustrate, but do not recognize, the newborn impulses which are superimposed on the true end-plate potential when curare has almost completely blocked neuro-muscular transmission.

CONCLUSIONS

The main conclusions that have been arrived at in this paper are shown diagrammatically in Fig. 25, in which nerve and muscle impulses are plotted on distance-time coordinates. Thus, on the ordinates, NE is the nerve path from the cathode of the stimulating electrodes to the end-plate E of a muscle fibre, and EM represents the path of an impulse discharged from E along the muscle fibre.

The course of the nerve impulse is shown tentatively by N_1E_1 in Fig. 25A, but the conduction velocity is not known in this profusely branching region of the motor nerve fibre, the curve drawn showing the fastest conduction velocity which is likely to obtain, i.e. a minimum nerve conduction time of 0.2 msec. (cf. section A3). The presumed slowing of the nerve impulses as they reach the fine terminal branches is shown by the curve in the line N_1E_1 . E_2M_1 shows the course of a muscle impulse which N_1E_1 causes to be discharged along a muscle fibre. It begins as a newborn impulse, which after slow propagation for about

SUMMARY

The main conclusions in this paper have been derived from a study of muscles (largely soleus, but occasionally tibialis anticus or peroneus tertius) prepared with their motor nerve cut down until the innervation is restricted to a narrow strip of muscle fibres with a sharply localized group of motor end-plates.

Investigation of the electrical responses produced in this innervated strip by a single nerve volley shows that:

(1) A single nerve impulse sets up the discharge of a single muscle impulse from the motor end-plate after a delay whose upper limit is never more than about 1 msec., and which is usually no more than 0.6 msec.

(2) Once set up this impulse travels in both directions along the muscle fibre at a uniform speed of 2.85–4.8 m./sec. for soleus and about 5–6 m./sec. for peroneus tertius and tibialis anticus.

(3) This impulse gives a brief negative potential wave at any point of the muscle fibre, but a triphasic or diphasic potential is always recorded because the active muscle fibres are embedded in an extensive conducting medium formed by the large inactive muscle mass. The negative potential wave is not more than 2 msec. in duration for soleus and 1.5 msec. for peroneus tertius and tibialis anticus, and it closely resembles in shape the nerve spike potential, the rise to the crest being about one-third of the total duration.

The following responses are produced either in the motor end-plate or in the soleus muscle fibre by a second nerve impulse at various intervals after the first. Similar responses are set up in the other muscles, but the critical times are shorter (see Table I).

(1) The earliest second impulse (at about 0.65–1.0 msec.) that can be set up in a motor nerve fibre only gives rise to a negative potential of the motor end-plate region relative to the rest of the muscle, the time course and size of this potential usually being practically unaffected by the impulse interval until this is lengthened beyond about 1.6 msec. The latent period of this potential, however, is much shorter at the longer impulse intervals, and experimental evidence suggests that this is due to lengthened conduction time of the early second nerve impulse. If this is so, the negative potential wave is set up by a nerve impulse with a delay of no more than about 0.7 msec., reaches its maximum about 1.5 msec. later, and declines to half about 2.5 msec. later still, usually having almost disappeared at 10 msec. after its origin. It has been

potential set up by N_2E_3 is shown to be only 80% of that produced by N_1E_1 .

In Fig. 25C the nerve volley interval N_1N_2 is at the shortest interval at which a second discharge of muscle impulses is evoked. The neuro-muscular delay for this discharge E_4E_5 is shown not to differ significantly from E_1E_2 , but there is a prolonged period of almost 0.8 msec. before the muscle impulse set up at E_5 grows to the fully propagated size, and as such travels at a uniform speed to M_3 . At such a critical nerve volley interval many newborn muscle impulses fail thus to grow up, their course being shown from E_5 to M_2 where, failing to spread any further, they die out. The end-plate potential set up by N_2E_4 begins at E_6 , and the effect of the rapid increase of end-plate negativity in helping the growth of the newborn impulses (cf. section E3) may be well imagined.

Finally, in Fig. 25D the nerve volley interval is 5.0 msec. The newborn impulse set up by the second volley is now shown to grow up to the fully propagated size even more quickly than that of the first volley. This has been attributed to the background excitatory effect produced by the end-plate potential set up by the first nerve volley, this excitatory effect now being evident on account of the recovery of the muscle from its relatively refractory state (section E3).

In section E3 it has been shown that a nerve impulse gives rise to two excitatory actions at the neuro-muscular junction.

(1) *The brief excitatory action by which newborn muscle impulses are set up.* Experiments to be described in later papers indicate that an intermediary reaction of the end-plates is involved. Clearly this excitatory action is analogous to the detonator response described for synaptic transmission [Eccles, 1936, 1937b]. It would thus seem that this detonator response can be distinguished from the newborn impulses, though, in the original experiments demonstrating the detonator response, summation of newborn impulses probably played a part [cf. Eccles, 1939].

(2) *The more prolonged excitatory action which is associated with the end-plate potential.* This is analogous to the central excitatory state and the N wave described for synaptic transmission. Presumably, too, the N wave and the associated excitatory state in smooth muscle [Eccles & Magladery, 1937] are closely related.

Thus the evidence presented in this paper further supports the view that the same two fundamental excitatory processes are involved in all quick transmissions across junctional regions [Eccles, 1936; 1937a, b].

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concluded that the motor end-plates are the only probable sites of the origin of this potential wave—hence it is called the end-plate potential. It is usually about 10% of the muscle spike potential.

(2) With impulse intervals from about 1.6 to 1.9 msec., the second nerve impulse sets up a small muscle spike potential at the end-plate region, but it is not propagated beyond this region and it fails to give rise to any detectable muscle contraction. The nerve impulse has set up a newborn muscle impulse (analogous to that recently described for nerve) which quickly dies out on account of its small size and the refractoriness of the muscle. Since this newborn muscle impulse may begin slightly before the end-plate potential, it must be independently produced.

(3) The longer the nerve-impulse interval, the larger is the newborn muscle impulse, and at intervals of about 1.9 msec. the newborn impulse is so large that, after a period of indecision up to 1 msec., it flares up to the fully propagated size and traverses the whole length of the muscle fibre which is then caused to contract. At still longer impulse intervals the newborn impulse is still larger, the growth to the fully propagated size being eventually as quick as with the response to the first impulse.

Comparison of the end-plate potentials set up in completely curarized and in normal muscle and, in addition, the action of small doses of curare and eserine show that a *single* nerve volley also sets up an end-plate potential which begins, if anything, slightly later than that due to the muscle impulses.

Thus a nerve impulse exerts two excitatory actions at the neuro-muscular junction. (1) Newborn muscle impulses are set up by a brief excitatory action probably no more than 1 msec. in duration and analogous to the detonator action described for synaptic transmission. (2) The much more prolonged end-plate potential is set up independently of the newborn impulses, but, if the growth of these impulses is sufficiently delayed, it appears to aid in their growth to the fully propagated size. It is analogous to the N wave and the associated central excitatory state of synaptic transmission, and analogous responses have also been described at the neuro-muscular junction of smooth muscle.

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THE EFFECT OF CHOLINE ON THE FATTY LIVER OF CARBON TETRACHLORIDE POISONING

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In a previous communication [Best, MacLean & Ridout, 1935] it was shown that when rats maintained on a diet low in lipotropic factors were poisoned with phosphorus, addition of choline to the diet had no effect on the degree of fatty infiltration or damage to the hepatic cells. On the other hand, the addition of choline increased the rate of removal of excess fat which was present in the livers of the poisoned animals.

Since it is well known that diets low in choline result in the accumulation of fat in the liver, it was not possible to differentiate between the liver fat which was present due to the lack of choline and that which was caused by phosphorus poisoning. Maintenance of animals on a diet with added choline, sufficient partially to counterbalance the effect of the low-choline diet, should result in a more marked difference in the amounts of fat present in the livers of the poisoned and control animals. This should make it possible to determine if the addition of choline exerts an effect on the fat which has accumulated as a result of the poisoning. This procedure was therefore adopted in the present investigation.

Carbon tetrachloride was used in the experiment to be described, since this material produces very fatty livers and the degree of poisoning is more easily controlled than with phosphorus.

EXPERIMENTAL PROCEDURE

One hundred and twenty-four male rats with an average weight of 228 g. were divided into three groups. All animals were maintained on the following basal diet which was low in choline and other lipotropic

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EXPLANATION OF PLATE I

Fig. 3A. Photograph showing three glass tube electrodes (outlined in black) encased in brass tubes and projecting through a wall of the animal box. Each brass tube is fixed in a universal joint outside the box so that adjustments can be made after the box is closed. The three electrodes are shown making contact with the active muscle strip of soleus, the lowest electrode being placed over the end-plate zone. The preparation is set up in a Brown-Schuster myograph around which the box has been built.

Fig. 3B. As in Fig. 3A but with the lid of the box in position. The anterior end of the cat is seen projecting from the left side of the box. Above may be seen the torsion wire myograph and the reflecting prism of the myograph system. The Zeiss dissecting microscope is in position for observing the electrodes through a glass window in a wall of the animal box, so that they can be manipulated under direct observation.

DISCUSSION

There is no significant difference in the amount of fat present in the livers of the two groups of animals poisoned with CCl_4 until the 8th day. Addition of a large excess of choline to the diet had no effect on the accumulation of fat due to the poisoning. On the 14th day the amount of fat present in the livers of the poisoned animals receiving 5 mg. choline for each 10 g. food is much greater than that present on the 8th day. On the other hand, in the group of animals which received 100 mg. choline for each 10 g. food, a large decrease in liver fat occurred during this interval. At 20 days the fat content of the livers of the poisoned animals receiving 100 mg. choline is almost normal, whereas the livers from a comparable group given only 5 mg. choline contain a large amount of fat. Five mg. choline for each 10 g. food were obviously insufficient to maintain the liver fat of unpoisoned animals at a normal level. The marked difference in the amount of fat present in the livers of groups I and II at all periods of the experiment shows that a large part of the excess fat present in the livers of group I was brought about by the treatment with CCl_4 . Addition of an excess of choline to the diet has resulted in the removal of almost all this excess fat within a period of 20 days.

The results of the examination of histological sections of liver taken from all groups of animals at 5, 8, 14 and 20 days were in general agreement with the chemical findings. There was no significant difference at the 5 and 8-day periods between the livers of groups I and III. At 14 days the livers from group I (receiving 5 mg. of choline) showed decidedly greater damage than those from group III which had received 100 mg. of choline. This difference was much more marked at 20 days, at which time the livers from group III presented a nearly normal appearance, whereas those from group I showed the presence of an excessive amount of fat.

The livers from group II appeared almost normal at 8 days, and at 14 days showed only a moderate accumulation of fat; there was, however, a wide variation in the appearance of individual livers. At 20 days livers from this group were very fatty; in fact it was not possible to distinguish between sections from groups I and II, although the chemical estimations showed that livers from group I contained about twice as much fat as those taken from group II. In this connexion it should be pointed out that when a liver contains a certain level of fat, it is not possible to determine by histological means either the presence of additional fat or the degree of degeneration of liver cells since these changes are masked by the vacuoles or fat globules which are present.

SUMMARY

Rats maintained on a diet low in lipotropic factors with an amount of added choline sufficient partially to prevent the increase in liver fat due to a low-choline diet develop very fatty livers during the 20-day period

factors: meat powder 10%, beef fat 20%, sucrose 63%, agar 2%, salt mixture 5%, vitamins A, B₁ and D.

Five mg. choline for each 10 g. food were given to the animals in groups I and II. Previous experiments had shown that this amount of choline prevents, to a considerable extent, the accumulation of fat due to a low-choline diet. The rats in group II received the same amount of

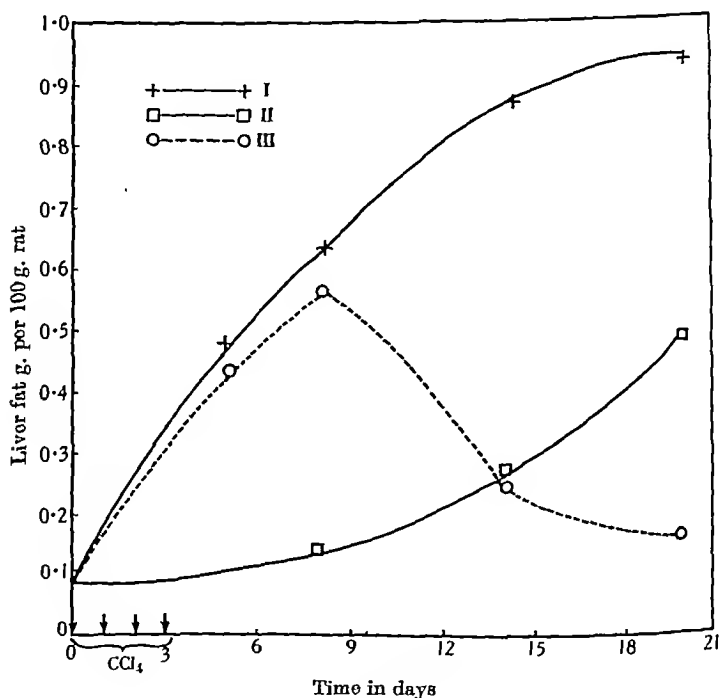


Fig. 1.

food as those in group I. One hundred mg. choline for each 10 g. food were given to the animals in group III. Groups I and III were given four subcutaneous injections of 0.8 c.c. CCl₄ on the first four days of the experiment. Animals were killed on the 5th, 8th, 14th and 20th days after the initial injection and the amount of fat present in individual livers was estimated by Liebermann's saponification method as modified by Leathes & Raper [1925]. The experimental results are shown in Fig. 1. Each point on the curve represents the average amount of fat obtained from the livers of twelve to fifteen animals.

DIET AND THE INSULIN CONTENT OF PANCREAS¹

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It is well known that fasting, or feeding diets rich in fat and poor in carbohydrate, leads to a change in the metabolism of sugar as judged by (1) the glucosuria following glucose administration, (2) the diabetic type of sugar tolerance curve when glucose is given, and (3) the absence of the normal rise in respiratory quotient after glucose administration. There is evidence that the administration of insulin at least partially restores the normal metabolism of carbohydrate. An excellent review of the relevant literature has recently been published by W. H. Chambers [1938].

We have reported in a preliminary communication [Haist, Ridout & Best, 1939] that a very definite change in the insulin content of pancreas may be produced by alterations in diet. In order to investigate this subject it is necessary to have available (1) an experimental animal which will ingest the diets provided and one from which all the pancreatic tissue can be removed without undue difficulty; (2) an extraction procedure which consistently gives optimal yields of insulin from pancreatic tissue; and (3) a method of testing which gives accurate results when relatively small amounts of insulin are available. The first requirements are satisfied when the Wistar rat is used as the test animal. A suitable method for the extraction of insulin is that outlined by Jephcott [1932] and by Scott & Fisher [1938*a*]. Satisfactory assays of the insulin content of the extracts are obtained by the mouse method of testing.

The results of these investigations which will now be described demonstrate, among other points, that a very definite decrease in the insulin content of pancreas is brought about by fasting or by the ingestion of diets rich in fat.

¹ The material in this and in subsequent papers on this subject will be incorporated in a thesis to be presented by one of us (R. E. H.) in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the University of Toronto.

following the administration of CCl_4 . Animals poisoned with CCl_4 and fed the same diet, with the addition of excess choline, have almost normal livers at the end of the same period. The results of this experiment strongly suggest that choline or other lipotropic factors are essential for the removal of the excess fat which accumulates in the livers of rats as a result of CCl_4 poisoning.

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reference to a standard curve. The characteristic dose-response curve for our mouse colony was obtained by injecting some 6000 mice with various dilutions of the standard solution. From 200 to 300 mice were used in assaying the potency of each extract. The animals were given dextrose as soon as convulsions appeared and those which recovered were used again after one week. For the most part, the insulin content of the pancreas is expressed in terms of units per group of ten rats and also as units per kg. initial body weight of rats. Since the amount of pancreatic fat is so variable, it was thought that any expression based on pancreatic weight would be less reliable. However, where portions of a homogeneous mixture of pancreatic tissue are compared, as in the test of the method, results are expressed as units of insulin per gram of pancreatic tissue.

Assay of pancreatic tissue

It will be appreciated that the method of preparation of insulin used in these experiments provides us with a relatively crude material. Nevertheless, it is perfectly adequate for determinations of activity and further purification would probably involve a loss of potency. Jephcott [1932] has shown that crude insulin added to minced pancreatic tissue can be recovered quantitatively by the extraction procedure we have used. This has been confirmed repeatedly by D. A. Scott in the Connaught Laboratories and by E. T. Waters in the Department of Physiology. It was decided that the best way to test our methods was to secure a homogeneous preparation of pancreatic tissue and conduct extractions and assays on weighed portions of this material. Two such preparations were used. Each was obtained by removing the pancreatic tissue from fifty rats. The first group consisted of normal animals, fasted overnight, while the second had been maintained for 7 days on a diet consisting only of beef fat, agar and vitamins A, B₁ and D. As each pancreas was removed it was dropped into liquid air and, when fifty had been collected, the tissue was ground to a very fine powder in a cooled mortar. The powder was thoroughly mixed and five aliquots were taken for extraction and assay. The results of these experiments, which are collected in Table I, demonstrate the consistency of the findings under these conditions. No further comment is required here except that it may be stated that *the variations on which we have placed significance are far greater than any which could be expected to result from the errors inherent in the method of extraction and testing.*

In practically all cases the solutions were tested on mice within a week after preparation. They were kept in the refrigerator during this

METHODS

In most of the experiments male rats weighing from 200 to 300 g. were used. They were from 100 to 200 days old. In any one experiment the rats were from the same age group and the initial weights of each group of ten animals were equal. The rats were kept in individual cages. The diet for each animal was weighed and the uneaten residue was recovered and weighed daily. Unless otherwise stated, the food was removed 14 hr. before pancreatectomy. Each test solution was made from the pancreatic tissue of ten animals. The rats were usually anaesthetized by the intraperitoneal injection of a solution of "sodium amytal". All the pancreatic tissue was carefully dissected from the anaesthetized animals and added to the extraction fluid immediately after its removal. The extraction fluid was made by mixing 750 c.c. absolute alcohol, 250 c.c. distilled water and 15 c.c. conc. HCl. Approximately 5-6 c.c. of this solution per gram of pancreas were used. The actual procedure for the preparation of the insulin-containing extract was as follows. The container with the required amount of extraction fluid was weighed before and after the addition of the pancreatic tissue. After the second weighing, the pancreas was thoroughly minced with scissors. The mixture was shaken at frequent intervals, allowed to stand overnight in the refrigerator, and was then filtered through cheese cloth. The solid material was pressed until nearly dry and re-extracted with the same volume of the acid solution. The mixture was allowed to stand for 2 hr. and was again filtered through cheese cloth. The two filtrates were combined, made just alkaline to litmus by addition of ammonium hydroxide and the total volume of the extract was measured. After filtering through Whatman no. 1 filter paper, five 9 c.c. aliquots were placed in 50 c.c. centrifuge thimbles. Fifteen c.c. of absolute alcohol and 25 c.c. of ether were then added to each thimble. These were placed in the refrigerator overnight. The next day the mixtures were centrifuged, the supernatant fluid discarded and the tubes drained. The precipitate in each tube was dissolved in isotonic saline (pH 2.5) and the solutions were combined and made up to a definite volume.

The potency of these solutions was estimated by the mouse method of assay. The procedure which we have followed was essentially the same as that described by Trevan & Boock [1926] and by Trevan [1927]. This test depends upon the relative number of mice convulsing when the standard and unknown solutions are administered under comparable conditions. The ratio of the potencies of the two solutions is obtained by

Effect of fasting

The effect of fasting on the insulin content of pancreas is shown in Table III. The total number of fasted rats was 130, and 110 control animals were used. All the animals had previously received a well-balanced diet. The average loss in weight of the fasted animals was 23 %

TABLE III. Effect of fasting on the insulin content of pancreas

No. of rats	Units of insulin per group of 10 rats		Units of insulin per kg. of initial body weight	
	Fasted* 7 days	Control	Fasted* 7 days	Control
20	11.1	20.0	3.9	6.9
20	11.0	19.0	3.8	8.8
20	13.5	28.4	4.0	7.9
20	16.6	28.8	5.0	8.1
10	16.2	—	4.9	—
20	11.9	20.5	3.3	5.8
20	16.0	23.9	4.5	6.8
10	17.1	—	4.8	—
20	16.6	31.4	4.4	8.4
20	15.2	28.8	4.1	7.7
20	11.1	29.7	3.0	8.1
20	12.2	29.7	3.3	8.1
20	14.5	31.4	3.9	8.4
Average	14.1	26.5	4.1	7.5

* Average loss in weight 23%.

of the initial value. Since there was considerable variation in the initial weights of the different groups, it is best to compare the values for the starved and control animals in each individual experiment. It is evident from these figures that fasting produces a definite decrease in the insulin content of the pancreas.

Effect of feeding fat or sugar

The results of an experiment designed to study this point are collected in Table IV. In the first experiment one group of fifty rats ate the balanced diet and a similar group consumed the fat diet *ad libitum*. The definite fall in the insulin content of the pancreas of animals receiving a diet rich in fat is evident. In the second experiment a paired feeding test was conducted on two groups of thirty rats each. The value for the sugar-fed animals, 18.8 units of insulin per group of ten rats, is below the average value for normal animals, but the insulin content of fat-fed animals receiving the same caloric intake as those which received sugar is much lower than that of the sugar-fed group. A very important point emerges from a consideration of the latter results. The weight loss in the

interval. Tests have shown, moreover, that under these conditions the change in potency of the solution is barely appreciable when 2 weeks are allowed to elapse between extraction and test.

TABLE I. Test of the method

Control diet			Fat diet, 7 days		
Aliquot no.	Weight of aliquot of pancreatic tissue g.	Units of insulin per g. of pancreas	Aliquot no.	Weight of aliquot of pancreatic tissue g.	Units of insulin per g. of pancreas
1	14.6	2.08	1	11.8	1.25
2	12.3	1.83	2	10.2	1.12
3	14.6	2.20	3	11.7	1.22
4	14.0	2.21	4	10.8	1.24
5	13.5	2.18	5	12.0	1.20
Average		2.10			1.21

Effect of anaesthesia

In order to determine the effect of certain anaesthetics upon the insulin content of pancreas, three groups of animals were studied. One group was anaesthetized with urethane, one with "sodium amytal" and the third was stunned. In each group half the rats were fasted overnight, and the other half were fasted for 7 days. The length of time under anaesthesia was the same for the urethane and "sodium amytal" groups. In the stunned animals, the pancreas was removed immediately. The results in Table II show that no significant difference exists between

TABLE II. Effect of anaesthesia on the insulin content of pancreas

		No. of rats	Units of insulin per group of 10 rats	Units of insulin per kg. of initial body weight
Urethane	Control	10	31.4	8.4
	Fasted 7 days	10	16.6	4.4
Sodium amytal	Control	10	28.8	7.7
	Fasted 7 days	10	15.2	4.1
Stunned	Control	10	31.4	8.4
	Fasted 7 days	10	14.5	3.9

the insulin contents of the pancreatic tissue of the three groups of animals. Blood sugars were determined upon the groups receiving urethane and "sodium amytal" by the Shaffer-Somogyi method [1933]. While the sugar content of the blood was definitely higher in the group which received urethane, this apparently produced no effect upon the insulin content of the pancreatic tissue. In most of the subsequent experiments "sodium amytal" was used as the anaesthetic.

which provided 41.8 cal. The group receiving the fat diet ate 4.8 g. per day which was equivalent to 38.9 cal. The loss of weight in the two groups is comparable but, as noted above, the insulin content of the pancreas of the two groups is quite different. These results, in addition to showing the fall in insulin content which occurs when diets rich in fat are used, demonstrate that carbohydrate tends to prevent the decrease.

Effect of various diets in fasted animals

In this experiment all the animals were starved for 7 days. They were then divided into various groups and placed on different diets. Three groups received sugar only, three fat only, and three were given a

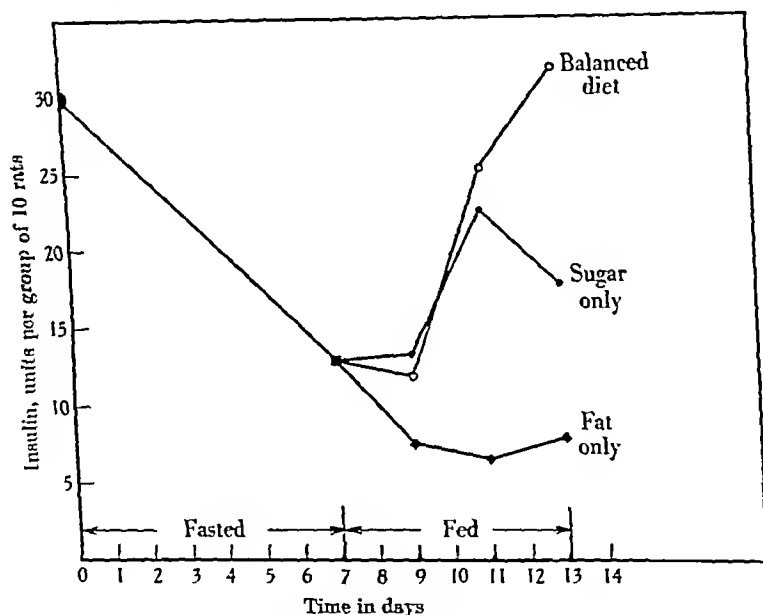


Fig. 1. Diet and the insulin content of pancreas.

balanced diet. All the rations provided approximately the same caloric intake and this was based on the food consumption of the carbohydrate-fed group. Groups were killed on the 2nd, 4th and 6th days. The insulin content of the pancreas was determined and the results are shown in Fig. 1. It will be quite evident that feeding fat alone does not lead to a restoration of the insulin content. On the contrary, there appears to be a significant further drop. Sugar alone leads to a return towards the normal value, while the adequate diet results in a complete restoration

TABLE IV. Effect on the insulin content of pancreas of diets containing only fat or sugar*

No of rats	Duration of exp. days	Loss in weight %	Diet	Units of insulin per group of 10 rats	Units of insulin per kg of initial body weight
50	7	3	Balanced†	28.9	8.8
50	7	13	Fat†	13.7	4.2
30	14	17	Sugar‡	18.8	6.5
30	14	20	Fat‡	10.9	3.8

* Fat or sugar actually constituted 90% of the material given. Agar, salt mixture, and vitamins A, B₁ and D made up the remainder.

† Rats ate *ad lib*.

‡ These two groups had the same caloric intake

group which received sugar only was 17% of the initial value, while that of the group receiving the same caloric intake in the form of fat was 20% of the initial value. The initial weights of the two groups were the same at the start of the experiment. *These figures show that weight loss alone is not the factor which is responsible for the change in the insulin content of the pancreatic tissue.*

It may be remarked here that when animals are placed on diets composed only of sugar, protein, or fat, the insulin content of pancreas falls in all cases. However, the caloric intake in these groups is not normal. The animals which are provided with protein eat so little that under-nutrition plays a large part in the results obtained. Even when this is involved, results of some preliminary experiments indicate quite definitely that the fall in insulin content is not as great in the group receiving protein as in the group where fat only is ingested. For example, in one series the control value was 31 units of insulin per group of ten animals, while the group which received sugar only gave a value of 22 units. In the protein-fed animals 18 units were present in the pancreatic tissue of ten rats, whereas those animals which received fat showed approximately 9 units per group.

In the first experiment described in Table IV the animals on the diet rich in fat ate as much as they desired. The weight loss in this group was 13% of the initial value after 7 days. It will be noticed that the decrease in the insulin content was as great as in the group which had been starved for the same period (Table III) although the loss of weight was not nearly so extensive.

In the second experiment described in Table IV the animals receiving sugar and those ingesting fat were fed in pairs so that each group had an equivalent caloric intake. The sugar-fed group ingested 11.6 g. per day

46% fat by weight was provided. The diet high in carbohydrate contained 69% carbohydrate, 15% protein, and 5% fat. The control diet consisted of 50% carbohydrate, 18% protein, and 16% fat. These diets contained a salt mixture, yeast, and cod-liver oil. The results of these experiments are summarized in Table V.

While there appears to be a decrease in the insulin content when the animals are fed the diet moderately rich in fat, the findings do not suggest that extensive depletion of insulin could be rapidly produced by diets of this type.

Vitamin B₁ deficiency

While we are contemplating a thorough investigation of the effects of deficiency of the various vitamins, our results thus far are confined entirely to a study of the lack of vitamin B₁. The diet was made up of beef muscle powder 10%, beef fat 20%, sucrose 62.7%, salt mixture 5%, agar 2%, choline chloride 0.3%, and vitamins A and D in the form of a cod-liver oil concentrate. One group received 16 µg. of crystalline vitamin B₁ for every 10 g. of diet. The rats in the first group ate freely while those in the second group were given the same amount of food as the animals which received the diet deficient in vitamin B₁. During the first 2 weeks 16.5 µg. of vitamin B₁ per rat per day were ingested, in the third week 10.9 µg. and in the fourth week 7.5 µg. were ingested. The results of the insulin determinations are given in Table VI and show

TABLE VI. Vitamin B₁ deficiency and the insulin content of pancreas

No. of rats	Diet	Units of insulin per group of 10 rats	Units of insulin per kg. of initial body weight
29	B ₁ deficient	12.0	9.4
29	B ₁ added	13.4	10.5

that, while in both the control and the test group there is a very definite decrease in the insulin content as compared with normal animals, the addition of vitamin B₁ had no effect. Presumably the decrease was due, in large part, to deficient caloric intake. These results are being extended.

Liver fat and the insulin content of pancreas

In some of the experiments in which diets rich in fat were fed, the rats developed fatty livers. It was therefore considered advisable to determine whether or not fatty changes in the liver might be associated with a change in the insulin content of pancreas. We had the opportunity to conduct tests on the pancreatic tissue from (1) rats poisoned with carbon tetrachloride, (2) rats poisoned with carbon tetrachloride and given

of the normal value within 6 days. At the end of 2 days, however, there was no indication of recovery. The results of this experiment furnish further evidence that loss of body weight may not be an important factor in the decrease in the insulin content of pancreatic tissue. Several sets of figures which illustrate this point are given in Fig. 2.

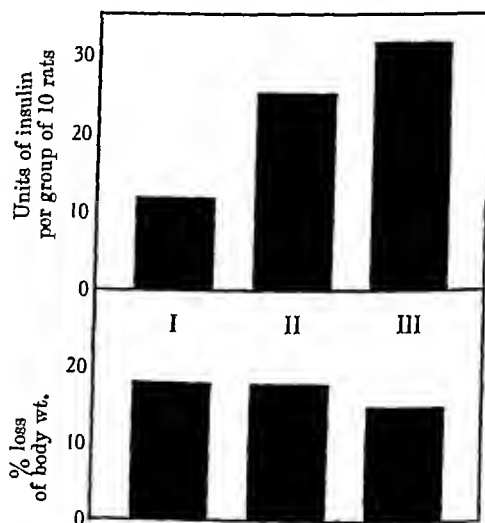


Fig. 2. Following a fast of 7 days, a balanced diet was fed for: I, 2 days; II, 4 days; III, 6 days.

Effect of diets moderately rich in carbohydrate and fat

In order to determine whether or not a diet *moderately* rich in fat would exert an effect on the insulin content of pancreas over longer periods of time, a diet containing 28% carbohydrate, 15% protein, and

TABLE V. Effect of moderately high fat and carbohydrate diets on the insulin content of pancreas

No. of rats	Diet	Duration of exp. months	Average weight per group of 10 rats		Units of insulin per group of 10 rats
			Initial g.	Final g.	
30	Control	2	1043	2345	26.7
30	Fat	2	1033	2373	20.1
60	Control	6	955	2661	29.9
59	Fat	6	959	2618	23.3
30	Control	3	737	2525	25.3
30	Carbohydrate	3	655	2246	23.9
18	Control	7	691	3337	25.0
67	Carbohydrate	7	613	2347	24.9

assumption that the insulin-producing tissue shares equally with the other tissues in this loss. The exact weight of the pancreatic tissue in rats is difficult to determine since the amount of fat closely associated with the pancreas may vary. *In the experiments where food was administered after fasting, the weight loss still remained extensive even in those animals whose pancreas showed a recovery of the normal insulin content (Fig. 2).*

The insulin content of the pancreas represents a balance between the rates of production and liberation of the hormone. A lowered insulin content such as we have observed after fasting or fat feeding may therefore be due to either diminished production or increased liberation or both.

Although no work has yet been done in our laboratory on the glucose tolerance of starved rats, or of rats fed on a diet rich in fat, it would appear from the results of other investigators on other species that the impaired carbohydrate tolerance occurs at a time when, in the light of our experiments, a decrease in the insulin content of the pancreas would be expected. As stated previously, the normal carbohydrate metabolism is partially restored by the administration of insulin [Cori & Cori, 1926, 1927; Dann & Chambers, 1930; Ellis, 1931]. Since the diminished carbohydrate tolerance in starvation and after feeding fat may be explained, in part at least, on the basis of decreased liberation of insulin, it would seem logical to assume that the low insulin content of pancreas which we have observed is caused by decreased formation rather than by increased liberation of insulin.

While no general relationship between the insulin content of the pancreas and the rate of insulin liberation has been established, there are experimental results which indicate that the liberation is related to the insulin content under certain conditions. It has been shown, for example, that in the permanent diabetes produced in dogs by the administration of anterior pituitary extracts, the insulin content of the pancreas is reduced to extremely low values [Campbell & Best, 1938]. Furthermore, in many diabetic patients the results obtained by Scott & Fisher [1938b] show very clearly that the insulin content of the pancreas is reduced well below the normal value. In these cases in which the insulin content of the pancreas is low the organism suffers from severe diabetes and is favourably affected by small doses of insulin. Under these circumstances it is reasonable to suppose that the low insulin content leads to a reduced rate of liberation. There is certainly no evidence at present that there is an increased rate of liberation and that the effect of this is overbalanced

choline, and (3) control rats receiving the same diet as those in the first group and a similar caloric intake. The results in Fig. 3 show clearly that the insulin content of the pancreas is not affected under these conditions when extensive changes in liver fat have occurred. Further details of these experiments are given in a paper by Barrett, Best, MacLean & Ridout [1939].

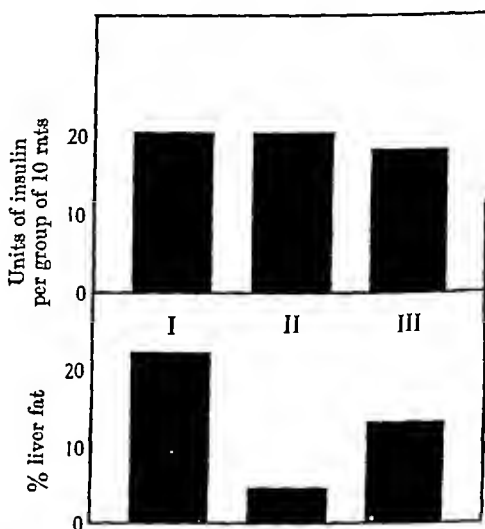


Fig. 3. I, animals poisoned with carbon tetrachloride; II, animals poisoned with carbon tetrachloride but receiving choline; III, control animals receiving same caloric intake as group I.

GENERAL DISCUSSION

It is obvious that the altered carbohydrate metabolism observed in starvation or as a result of feeding diets rich in fat might be produced by factors affecting the production of sugar, the rate of utilization, or both. Factors affecting the production of sugar in the liver or the rate of utilization in the tissues might act directly on these structures, or might exert their effects through one or more of the endocrine glands. At present there are very few facts available which enable one to decide which of these mechanisms are in operation. Our results show conclusively that in rats one definite finding is present under these conditions, namely, that there is a decrease in the insulin content of pancreatic tissue.

In many of the experiments reported the loss of body weight was considerable. In all the experiments, however, the change in insulin content of the pancreas was definitely greater than that which might be expected merely from loss of weight if one makes the unjustifiable

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by opposing factors. On the other hand, in those clinical cases suffering from liberation of excessive amounts of insulin, i.e. hyperinsulinism, it has been established that the tumours of islet tissue may contain abnormally large amounts of the hormone. For example, in one of the recent cases reported by Campbell, Graham & Robinson [1939] in which D. A. Scott estimated the insulin content of the tumour, the concentration of insulin was 8 units per gram. The patient suffered from a very definite hyperinsulinism which was alleviated by removal of the tumour.

We have merely cited evidence which suggests that under certain conditions there is a relationship between the insulin content of pancreas and the rate of insulin liberation. It is quite possible that under other conditions this relationship may not hold or may be completely obscured by compensatory physiological adjustments. We are postponing a full discussion of the significance of our findings until further studies on the insulin content of pancreas have been made. It may be mentioned here, however, that research along this line will certainly throw further light on such problems as the mechanism of the effect of the undernutrition treatment of diabetes [Allen, Stillman & Fitz, 1919], the role of dietary substances in the aetiology of diabetes [Himsworth, 1935], the influence of obesity on the diabetic state [Newburgh, Conn, Johnston & Conn, 1938], and the use of diets very rich in fat in the treatment of hyperinsulinism.

SUMMARY AND CONCLUSIONS

1. Fasting or the feeding of diets rich in fat produces a very definite decrease in the insulin content of the pancreas.

2. While an abnormally low caloric intake may be one of the factors producing this result in the animals given the diet rich in fat, it is not the only one, since those receiving a certain caloric intake as fat show a much greater decrease in insulin content than those provided with the same caloric intake in the form of carbohydrate.

3. The insulin content of the pancreas, depleted by fasting, is restored within 6 days to a normal value by feeding a well-balanced diet. Carbohydrate alone effects a partial restoration but fat produces no rise in insulin content.

4. The results of preliminary studies on the effect of (1) short periods of anaesthesia and (2) vitamin B₁ deficiency do not suggest that these conditions affect the insulin content of the pancreas in a specific manner.

5. The possible relationship between the low insulin content of the pancreas and the altered carbohydrate metabolism of animals which have been fasted or fed diets rich in fat is discussed.

Group V. 15 pregnant ewes fed basal ration supplemented with oil-cake meals.

VI. 10 pregnant ewes fed to a fat condition.

VII. 15 pregnant ewes fed to a fat condition, then reduced in weight by a reduction in the ration in late pregnancy.

All the rations contained adequate supplies of calcium and phosphorus in balanced amounts.

Feeding. The basal ration was that used in the previous year's experiments [Fraser, Godden, Snook & Thomson, 1938]. The supplements of starch, maize and oil-cake were fed only when the results of blood analysis in an individual sheep showed that the blood contained 20 mg. or more of total ketone per 100 c.c. The sheep were individually fed.

Analytical methods. These were the same as used in 1937-8 [Fraser *et al.* 1938].

RESULTS

Group I. Ten barren ewes fed basal ration

The barren ewes in this group received the basal ration under the same conditions as the pregnant ewes in the other six groups.

After being fed the basal ration for 3 months, there was an average loss in body weight for the group of 10 lb. Some ewes lost considerably more; thus two lost as much as 24 and 21 lb. respectively. Nevertheless, they remained in good health throughout the experiment and no ketone bodies were found in the blood of any of them.

In the last month of the experiment the ewes were fasted for 2 days. This had no significant effect on the blood sugar level (average 46 mg./100 c.c. before and 42 mg. after the fast), and ketone bodies were not present in the blood. The ewes showed a fairly uniform loss in body weight during the fasting period (average 4 lb., range 3-5 lb.). The fast did not lessen the appetite of the ewes as they ate up all their feed immediately it was offered to them. This is in contrast to the behaviour of pregnant ewes under similar conditions.

Group II. Fifteen pregnant ewes fed basal ration

Most of the ewes in this group gained in weight until the last few weeks of their pregnancy, nine being heavier on 14 February than when the experiment began in November. At this time four had gained 10 lb. or more in weight. This is a distinct contrast to the average loss of 10 lb. of the barren ewes (group I) on the same ration.

KETONAEMIA IN PREGNANT EWES AND ITS
POSSIBLE RELATION TO PREGNANCY DISEASEBY A. H. H. FRASER, W. GODDEN, L. C. SNOOK
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In 1938 we described experiments designed to test the influence of various dietary factors upon the incidence of ketonaemia in pregnant ewes. The results suggested that the occurrence and severity of ketonaemia was inversely correlated with the caloric value of the diet, and that it was both more frequent and more severe in multiple than in single pregnancies. We also discussed the possible relationship of our experimental findings to the disorder of pregnant ewes called "pregnancy" or "twin" disease as it occurs in the field.

Further evidence is now presented of an inverse correlation between a caloric deficiency of the diet and the incidence of ketonaemia in pregnant sheep. The possible significance of our experimental findings to a clearer understanding of the diseases of pregnant sheep in the field is also more fully discussed.

METHODS

Ninety aged Greyface ewes were used. Most of these animals had been utilized for similar work in the previous year, ewes of comparable age and type being used for necessary replacements. The animals were divided into seven groups¹ which were as follows:

- Group I. 10 barren ewes fed basal ration.
- II. 15 pregnant ewes fed basal ration.
- III. 15 pregnant ewes fed basal ration supplemented by maize starch.
- IV. 10 pregnant ewes fed basal ration supplemented by flaked maize.

¹ We are indebted to Dr J. F. Tocher, University of Aberdeen, for advice in randomizing the individuals throughout the various groups.

individual sheep in groups III, IV or V became ketonaemic to the extent that its blood contained 20 or more mg. of ketones per 100 c.c. of blood, supplements were fed in addition to the basal diet. In group III the supplement fed was maize starch, a reasonably pure form of carbohydrate obtainable economically in large quantities; in group IV it was flaked maize, a concentrated carbohydrate foodstuff such as might be used in farming practice; in group V it was a mixture of protein-rich meals (linseed and earthnut), in order to discover whether protein had any special effect other than its caloric value in the cure of ketonaemia. The starch and protein equivalents of the basal and supplemented rations are shown in Table II.

TABLE II

Group		Total daily intake (lb.)	
		Starch eq.	Protein eq.
III	Basal ration as fed to groups I and II	0.65	0.09
IV	Basal ration + 0.5 lb. maize starch	1.15	0.09
V	Basal ration + 0.6 lb. flaked (cooked) maize	1.15	0.13
	Basal ration + 0.7 lb. protein-rich meals	1.17	0.43

Some difficulty was experienced in feeding these supplements, since the appetite of ketonaemic ewes is poor. This difficulty was most marked in the case of the unpalatable maize starch, least so with the palatable flaked maize. Nevertheless, the general result was perfectly definite. Where the supplement was eaten the condition of the ewe improved, the degree of ketonaemia was reduced, and the protein food was no more effective than the carbohydrate foods in producing these results. In Table III are summarized the changes in body weight, blood ketones and in blood sugar produced by supplementary feeding over a 14-18 day period. The corresponding changes in group II, which received no supplement during the same period, are appended for comparison.

TABLE III. Changes produced by 14-18 days' supplementary feeding.
Average values, groups III, IV and V

Basal ration	Change in body weight lb.	Blood constituents, mg./100 c.c.			
		Total ketones		Sugar	
		Before	After	Before	After
+ starch (seven ewes)	+0	29	7	29	40
+ flaked maize (eight ewes)	+5	35	5	29	50
+ protein-rich meals (eleven ewes)	+5	25	10	30	48
Control group for comparison (basal ration only)					
Group II, eight ewes with two or more lambs	-3	23	44	27	23

During the last few weeks before parturition, however, many of the pregnant ewes showed a loss in weight. A summary of the progressive changes in weight and of blood sugar and ketones is shown in Table I.

TABLE I. Progressive changes in group II, average values

Date	Live weight lb.	No. ewes	Blood constituents, mg./100 c.c.	
			Total ketones	Sugar
1. xii. 38	144	15	0	45
20. i. 39	147	8	2	44
	144	7	10	32
28. i. 39	147	3 (single preg.)	0	34
	145	12 (multiple preg.)	18	29
8. ii. 39	148	3 (single preg.)	2	33
	145	12 (multiple preg.)	29	23

Of the fifteen ewes in this group (II), three bore single lambs. These three ewes showed no signs of actual illness although in very poor condition at the end of pregnancy. The maximum values for blood ketones in those ewes carrying single lambs were 18, 18 and 26 mg./100 c.c., whereas the lowest maximum for ewes with multiple pregnancies was 28 mg.

Of the remaining twelve ewes which were pregnant with two or more lambs, four died, one gave birth to lambs which had been dead *in utero*, one was slaughtered for carcass examination when very ketonaemic (48 mg. total ketone/100 c.c. blood), and five of the remaining six gave birth to poor lambs and had very little milk.

In two cases death was preceded by a syndrome very similar to that seen in field cases of pregnancy disease, progressive hypoglycaemia and ketonaemia being succeeded by loss of appetite, blindness, and coma. On the day of death, the blood of one of these two ewes contained 99 mg. total ketone/100 c.c. and only 12 mg. sugar. Corresponding figures for the other ewe were 82 and 17 mg. Autopsy showed no gross changes except emaciation and fatty infiltration of the liver (64 and 65 % fat in the dry matter).

A third ewe died after giving birth to two dead lambs; the fourth ewe died 3 weeks after lambing.

Groups III, IV and V

These three groups may be reviewed conveniently together. In each group the initial feeding was the same as in groups I and II—that is to say a basal ration on which barren ewes lost weight and on which pregnant ewes carrying more than one lamb became ketonaemic. When any

is group which did not develop significant ketonaemia bore a single lamb.

One ewe aborted and died 5 weeks later. Another ewe died with symptoms suggestive of pregnancy disease. Post-mortem showed no gross changes except moderate emaciation and a pale, friable liver which contained 59 % fat in the dry matter.

DISCUSSION

The results of our experiments in the winter of 1938-9 in general confirm those we have already published (1938).

The suggestion that the caloric value of the diet is inversely correlated with the frequency of occurrence and the severity of ketonaemia in pregnant ewes has been confirmed by further and more exact experimentation. It is quite clear from the results of our experiments that ketonaemia of pregnant ewes can be produced at will by a diet insufficient to permit the normal increase of weight which occurs in the latter part of pregnancy, and that the ketonaemia so produced can be removed by the feeding of a pure carbohydrate, such as maize starch, or more easily by more palatable foods usually regarded as carbohydrate concentrates, such as flaked maize.

The significance of our experimental results to the full understanding, prevention, and cure of pregnancy disease as it is known to occur in sheep farming is naturally a much more debatable question. To presume that all outbreaks of pregnancy disease in commercial flocks are due to a caloric deficiency of the diet would be unjustified on our experimental evidence, nor would such a presumption be in accordance with the results of practical observation. We have succeeded in producing experimentally a condition of ketonaemia in pregnant ewes similar to if not identical with that seen in field outbreaks of pregnancy disease. But our experiments do not exclude the possibility that other causes, not necessarily all nutritional, may lead to the same result.

It is obvious, for example, that where appetite is lost, under-nutrition follows, no matter what the cause of the loss of appetite. For example, a mineral deficiency in the diet must result in under-consumption of all dietary constituents, since loss of appetite is an early sequel to mineral deficiency.

Again, any toxæmia or infection which led to a temporary loss of appetite might result conceivably in ketonaemia of pregnant ewes while without serious effect on barren sheep, since the results of our experiments have shown that even a short period of food deprivation or loss of appetite

Group IV. Ten pregnant ewes fed to a fat condition

In the experiments of the previous year 1937-8 [Fraser *et al.*] the ewes which were fed to overfatness throughout gestation completed uneventful pregnancies and at no stage became hypoglycaemic or ketonaemic. In 1938-9, however, in contrast to the previous year, four of these overfat ewes lost their appetites during the last few weeks of pregnancy, ceased to gain in weight and became markedly ketonaemic. The respective maximum values for blood ketones were 35, 44, 52 and 56 mg./100 c.c. blood; the corresponding blood sugar figures being 32, 54, 27 and 30 mg./100 c.c. The reason for this loss of appetite in certain of these overfed ewes is obscure. The six ewes which continued to gain in weight maintained high blood sugar levels and had insignificant amounts of ketone bodies in their blood. One ewe in this group died on 6 February 1939. This ewe carried only one lamb and was never hypoglycaemic or ketonaemic. The post-mortem findings showed an acute enteritis and not pregnancy disease.

Group VII. Fifteen pregnant ewes fed to a fat condition, then reduced in weight by a reduction in their ration during late pregnancy

In the experiments of 1937-8, a group of overfat, pregnant ewes near term were subjected to a 2 days' fast, followed by 7 days on quarter rations. The fast produced certain symptoms of blindness and coma suggestive of pregnancy disease, and a number of the ewes later became hypoglycaemic and ketonaemic, the ketosis disappearing when the full ration was again being consumed.

In 1938-9, the experiment was repeated with certain modifications in that two periods of fasting were imposed, one in early and one in late pregnancy, and that following the second fast the ewes were kept on reduced rations until they lambed.

The ewes reacted to the 2 days' fast in early pregnancy (4-6 weeks after conception) in the same way as barren ewes (group I). They lost weight but did not become either ill or ketonaemic. The pregnant ewes, however, only gradually regained their appetites when food was made available following the fast.

The 2 days' fast and subsequent under-nutrition at 3-6 weeks before lambing produced more marked effects. Several of the ewes became ill with symptoms suggestive of pregnancy disease, lassitude, blindness, inability to rise, unsteadiness of gait. The ewes became ketonaemic, those nearest term showing the highest blood ketone figures. The only ewe in

The value of the supplementary feeding appeared to be entirely dependent on its caloric value. Starch, when readily eaten, was just as efficient in effecting improvement as flaked maize or a protein-rich meals mixture.

The significance of these experimental results in relation to field outbreaks of pregnancy disease in sheep is discussed.

REFERENCE

Fraser, A. H. H., Godden, W., Snook, L. C. & Thomson, W. [1938]. *J. Physiol.* 94, 346.

produces serious results, including severe ketonaemia in ewes carrying twins or triplets and nearing term, without having any noteworthy effect on ewes carrying twins in early pregnancy, on ewes carrying single lambs throughout pregnancy, or on barren ewes at any time whatsoever.

Ketonaemia or pregnancy disease in ewes carrying more than one lamb may possibly not be a disease entity, but rather the characteristic pathological response of a heavily pregnant ewe to a variety of dietary or other factors. It is conceivable that in every case the immediate cause of the ketonaemia might be a caloric dietary deficiency, but the predisposing causes might well include many factors leading to insufficient intake of food, factors as diverse as a severe snow-storm or a mild toxæmia

SUMMARY

Experiments are described concerning the relation of food intake and the onset of ketosis in pregnant ewes. Ninety aged Greyface ewes, individually fed, were used.

Barren ewes kept on a submaintenance ration for 3 months did not exhibit any symptoms of ill-health, nor did they become hypoglycaemic, ketonaemic or lose appetite when, at the end of this period, a 2 days' fast was imposed.

Ewes fed to overfatness and kept in close confinement throughout gestation did not exhibit symptoms of ill-health, but near term partial loss of appetite was observed and those ewes carrying more than one lamb became ketonaemic. This ketosis had no harmful effect. Near term, the pregnant ewe appears particularly susceptible to ketosis.

Pregnant ewes kept during gestation on a basal ration, quantitatively inadequate to permit of reasonable increase in body weight, became hypoglycaemic and ketonaemic. Where no change was made in the ration the ketonaemia became progressively worse until death or term.

Marked hypoglycaemia or ketonaemia did not necessarily produce symptoms of ill-health. Ketonaemic ewes, however, carrying more than one lamb, tended to lose appetite, this being followed by a rapid increase in the degree of ketonaemia and loss in body weight. In eight animals this loss of appetite was succeeded by coma and death. Autopsy revealed emaciation, multiple pregnancy and fatty infiltration of the liver.

Hypoglycaemic, ketonaemic ewes receiving additional food in the last 2-4 weeks of gestation showed a rapid improvement in condition, the blood sugar level returning to normal, ketone bodies disappearing from the blood, and an uneventful parturition taking place.

examination of the ovaries was carried out usually 48, and sometimes 4 or 72 hr. after the injections. For the microscopical examination the ovaries were fixed in Bouin's fluid and stained with haematoxylin and eosin. All injections were made into the ear vein.

RESULTS

(a) *Picrotoxin*. The dose of picrotoxin was 0.9-1.1 mg./kg. body weight: this was lethal in about 20 % of the cases and convulsive in about 75 %. An attempt to prevent death by giving a small dose of ether whenever the convulsions began to involve the respiratory muscles, was made in all instances, but was not always successful.

Table I summarizes our results on the rabbits injected with picrotoxin only. The rabbits showing convulsions are treated separately from those in which salivation, strange posture and excitement were the only signs

TABLE I

Ovulation observed	Convulsions present				Convulsions absent			
	Follicles enlarged	Follicles haemorrhagic	Death from injection	No reaction of ovaries	Ovulation observed	Follicles enlarged	Follicles haemorrhagic	No reaction of ovaries
135 (ovulation imminent)	158	141	136	143	151	148	142	153
139	—	145	150	183	—	206	144	209
140	—	146	152	195	—	207	210	—
155	—	184	154	196	—	216	215	—
211	—	203	176	197	—	—	—	—
217	—	—	200	204	—	—	—	—
—	—	—	212	205	—	—	—	—
—	—	—	213	208	—	—	—	—
—	—	—	—	214	—	—	—	—
0	1	5	8	9	1	4	4	2

In all animals included in this table the dose of picrotoxin was 0.9-1.1 mg./kg. With the exception of the rabbit in which the reaction is described above as "ovulation imminent" the ovaries were examined 48 or 72 hr. after injection. The index number of each rabbit is given in the columns above, and in the last row are the numbers of animals which showed the effects described at the head of each column.

exhibited. If one discards those dying from the injections, there remain twenty-one rabbits which survived convulsions: six of these ovulated, and six showed batches of enlarged, cystic or haemorrhagic follicles, reactions which are usually considered as proof of subliminal stimulation of the ovary by the anterior lobe hormone. The nine rabbits which showed no signs of ovarian reactions were found to be either pseudo-pregnant, or not on heat as judged from the colour of the uterus and vulva. Whereas in this group of twenty-one animals six ovulated, one only did so in the

THE OCCURRENCE OF OVULATION IN THE RABBIT AS A RESULT OF STIMULATION OF THE CENTRAL NERVOUS SYSTEM BY DRUGS

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THREE years ago Marshall & Verney [1936] reported the results of experiments in which the effects of electrical stimulation of the central nervous system on the occurrence of ovulation in the rabbit were investigated. It was shown that ovulation and pseudo-pregnancy could readily be induced by these means, and favour was given to the view that the electrical stimulus produced these sexual changes by the functional intermediation of the anterior pituitary body. An attempt to produce ovulation in the rabbit on heat by the action of certain drugs which might conceivably, by direct or indirect means, bring about an increased activity of the anterior pituitary, proved unsuccessful, the drugs chosen being pilocarpine, eserine, acetylcholine and adrenaline.

It seemed to us of interest to see whether ovulation in the rabbit on heat could be induced by drugs with convulsive or other stimulating action on the central nervous system, thereby initiating by chemical means a train of events similar to that produced by an electrical stimulus. With this object in view we have used the following drugs: strychnine, apomorphine, β -tetrahydronaphthylamine, ergometrine, carbaminoylcholine hydrochloride, coriamyrtin and picrotoxin.

METHOD

The does were isolated for at least four weeks before use, and in order to prevent the occurrence of single haemorrhagic follicles, frequently found in such rabbits, more than half the animals were copulated with a vasectomized buck about 22 days before experiment. Post-mortem

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been found ineffective by Marshall & Verney [1936]. "Doryl" (carbaminoylecholine HCl), tested on rabbits 163 and 165, is another instance of an ineffective parasympatheticomimetic drug. The larger doses of this drug were preceded by injections of atropine, since they otherwise would have proved lethal. This procedure, however, is open to the criticism that the atropine may have interfered with the ovulation: indeed, Foster, Haney & Hisaw [1934] report that after injections of atropine the mating of does fails to produce ovulation. In our experience, however, ovulation may occur even when doses of atropine as high as 20 mg. are given about 10 min. before the mating, but difficulties arise from the fact that such treatment is frequently followed by a refusal to mate.

An attempt to test the effect of atropine on ovulation produced by picrotoxin gave inconclusive results, since the convulsive and lethal effect of the drug was increased by the atropine. It was necessary, therefore, to give less picrotoxin, i.e. 0.8 mg./kg. in the presence of atropine: in spite of violent convulsions, none of the rabbits so treated ovulated. Since this dose of picrotoxin does not produce ovulation in the absence of atropine, its failure to do so in the presence of atropine may be the simple result of the smaller dose of picrotoxin. These results may, however, be regarded as a further indication that no correlation exists between the convulsive action of picrotoxin and its effect on the ovaries.

Another example which shows that ovulation is not a simple consequence of any kind of convulsions is given by the result of similarly conducted experiments with strychnine (rabbits 133 and 134) and with coriamyrtin. The negative results with the latter drug are somewhat striking, since both picrotoxin and coriamyrtin appear to have similar actions on the central nervous system (see Swanson & Chen [1936]). Moreover, the effects both of apomorphine in a dose which produced violent general excitement (rabbits 147 and 149), and of two drugs which act on the sympathetic centres (β -tetrahydronaphthylamine (rabbit 156), and ergometrine (rabbits 159 and 160)), proved equally negative in this regard.

DISCUSSION

Picrotoxin, given intravenously to rabbits on heat, may produce follicle growth, follicle haemorrhages or ovulation. The absence of response to injections of a series of other drugs with a stimulating action on the central nervous system may be of significance: no definite conclusions from such negative experiments can, however, be drawn, since the experiments with picrotoxin have shown that positive results are only obtained within a very limited range of dosage and when the does are

other group (eleven animals) comprising those rabbits which did not show convulsions. With the exception of two rabbits, however, which were not on heat, all the others showed follicle growth or haemorrhage: the occurrence of convulsions is not, therefore, a condition necessary for the appearance of an ovarian reaction.

In those rabbits in which ovulation occurred, between three and five ovulating follicles were usually present in each ovary: in this respect the picture was the same as in normally induced ovulation. Occasional haemorrhages, however, into single follicles indicated that these ovulations were not entirely normal. Moreover, the time course of the pharmacologically produced ovulation was always abnormal: if the rabbits (see no. 135, Table I) were killed 24 hr. after injection, ovulation was only imminent, and not before 48 hr. after treatment with the drug was the process certainly completed. Compared with the natural latency of 10 hr. between copulation and ovulation, the period between chemical stimulus and ovarian reaction is very much longer, longer even than that found by Marshall & Verney [1936] in their experiments on the production of ovulation by electrical stimulation of the central nervous system.

(b) *Other substances.* A series of other drugs was tried in order to discover whether any substance other than picrotoxin would produce

TABLE II. No ovarian response was seen to these drugs when given in the dosage shown

No. of rabbit	Kind and dose of drug	mg./kg.	General effects
133	Strychnine HCl	0.15	Excitability
134	Do.	0.17	Convulsions
147	Apomorphine HCl	10.0	Violent excitement
149	Do.	10.0	Do.
156	β -Tetrahydronaphthylamine	12.0	Excitement and rise in temperature
159	Ergometrine	2.0	Exophthalmos, dilatation of the pupils, increase in respiratory rate and in temperature
160	Do.	2.0	Do.
163	"Doryl" (carbaminoylecholine HCl)	0.02	Salivation, defaecation, dilatation of ear vessels
165	"Doryl" (after 5.0 mg. atropine sulphate) followed by "Doryl" (after 6.0 mg. atropine sulphate)	0.05	—
168	Picrotoxin (after 20 mg. atropine sulphate)	0.8	—
169	Do.	0.8	Convulsions
170	Do.	0.8	Do.
171	Do.	0.8	Do.
172	Coriamyrtin	0.25	Do.
174	Do.	0.25	Do.

ovulation in does on heat. The results are summarized in Table 2: as will be seen, they were entirely negative. Adrenaline and a number of stimulants of cholinergically innervated effector organs had previously

THE AIR STREAM IN THE LUNG OF THE FOWL

BY JAS. D. P. GRAHAM

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THE exact mechanism of respiration in birds has for long been a matter of controversy. Investigators give support to one or other of two theories. The first of these states that air passes through the lungs into the air sacs by the main bronchi on inspiration and is expelled from the sacs on expiration by a different route, the sacs acting as bellows to ventilate the lungs. The rival theory states that the air sacs play no active part in respiration and that the route followed by the air current on inspiration does not differ from that on expiration.

Barclay, Franklin & Macbeth (1938 *a*, *b*) described a technique of insufflation of the lungs of living mammals with radio-opaque dusts, followed by X-ray examination. This delineated clearly the bronchial tree and incidentally recorded the course of the air stream in the lungs. This technique suitably modified and applied to a series of pigeons and fowls allowed of an investigation of the direction of the flow of air during respiration in birds, and thus of deciding in a physiological manner which of the two current theories should be accepted.

In the bird the lungs are small in relation to the body. Dorsally they are firmly attached to the ribs. In front they are limited by the membranous or false diaphragm, so that they are relatively non-expansile. The trachea divides into two mesobronchi which take a sinuous course through the lung substance and open into the abdominal and posterior thoracic air sacs on either side. From the mesobronchi arise a series of large entobronchi running ventrolaterally towards the false diaphragm. The first of these entobronchi opens into the cervical air sac and the third into the interclavicular and anterior thoracic air sacs. The opening into an air sac is known as a primary ostium. The entobronchi are continued into the parabronchi which pass through the lung substance and connect up again with the mesobronchus. From the parabronchi arise a multitude of parabronchial air capillaries where gaseous exchange can occur. Around the primary ostium in each sac are situated the openings of a series of fine recurrent bronchi which pass from the sac to connect with the parabronchial air capillaries.

fully on heat. Though most of the drugs were given in the highest amounts compatible with survival, the absence of response might be due to failure in finding the optimal conditions for their action. The most reasonable interpretation of our results, however, would appear to lie in a more elective action on the innervation of the anterior lobe of the pituitary by picrotoxin than by the other drugs whose action in this regard we have investigated.

SUMMARY

Picrotoxin in a dose of 0.9–1.1 mg./kg. was injected intravenously into rabbits on heat. Follicle growth, development of batches of cystic and of haemorrhagic follicles, or ovulation ensued in a large number of animals so treated. No ovarian responses were obtained to the injection of a series of other substances, most of which were stimulants of the central nervous system.

We are indebted to Dr G. H. A. Clowes and Messrs Eli Lilly and Co. for the coriamyrtin used in our experiments.

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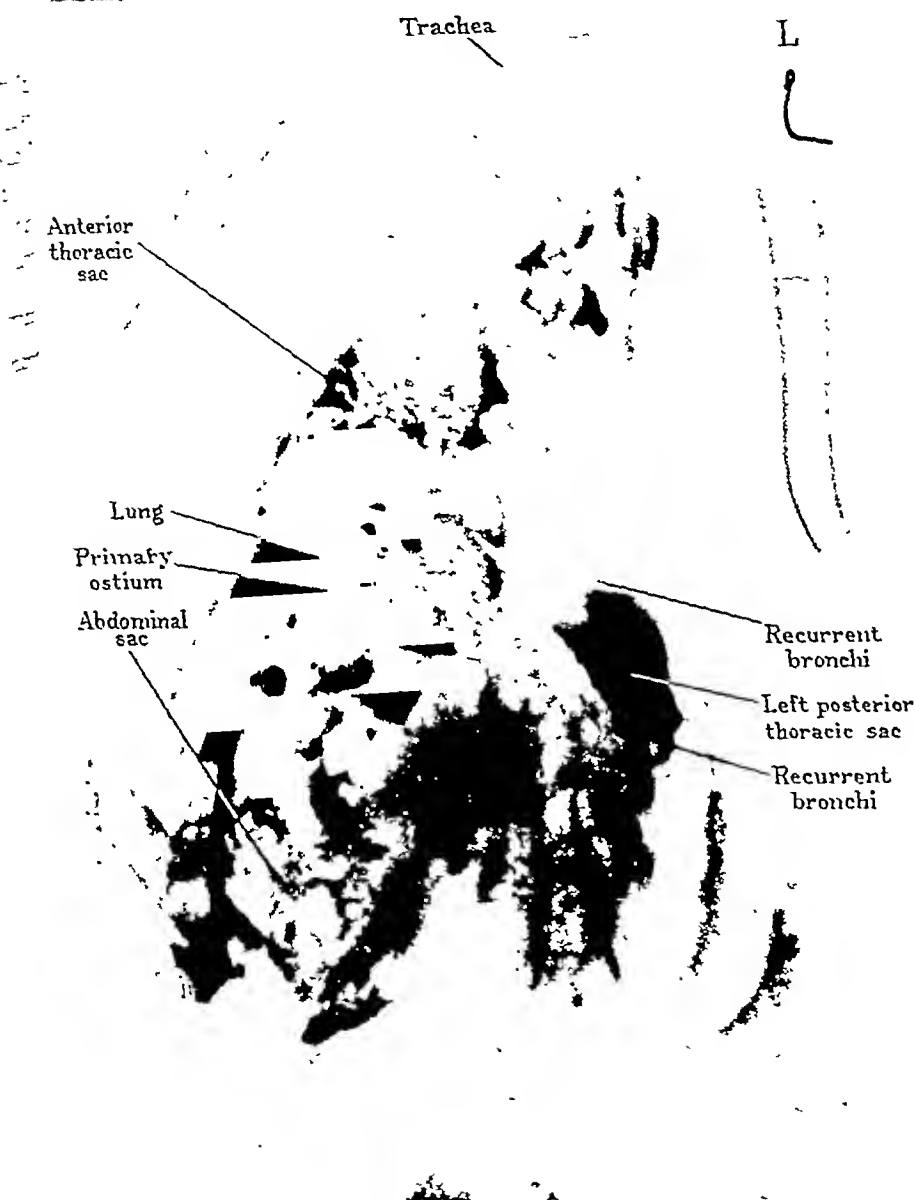


Fig. 2. Fowl, 1.2 kg. weight. 1.5 grains nembatal intramuscularly. Skiagram of respiratory apparatus of fowl, insufflated with powdered bismuth carbonate. Right side: bismuth powder in trachea, mesobronchus, entobronchi and abdominal and thoracic air sacs as a result of inspiration. Left side: bismuth powder in posterior thoracic air sac and spreading into parabronchi via recurrent bronchi as a result of expiration.

EXPERIMENTAL

Three hours before operation a fowl was given 5 mg. atropine sulphate intramuscularly in order to inhibit the bronchial secretions. Two hours later 1.5 grains nembutal was given intramuscularly. The bird was fixed on a warm operating table and the breast and neck plucked. The pectoral muscles were reflected through a midline incision and one and a half inches of the sternal plate removed, leaving the clavicular arch intact

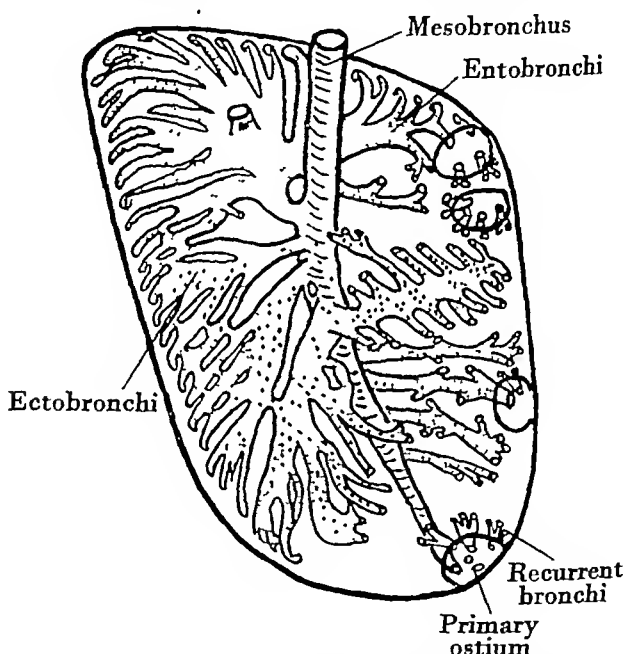


Fig 1 Diagram adapted from Locy & Larsell [1916] of lung of the fowl seen from the dorsal aspect. The origin of entobronchi and ectobronchi from the mesobronchus, and the position of the recurrent bronchi in relation to the primary ostium is clearly shown. This figure may be compared with Fig 2.

The upper border of the heart was drawn down and a clip placed on the left mesobronchus immediately below the bifurcation of the trachea. The opening in the thorax was temporarily closed and a tracheotomy performed high up in order to avoid interfering with the cervical air sac. A small insufflator with a fine bore nozzle was introduced into the trachea, care being taken not to block the air passage, and a puff of powdered bismuth carbonate introduced during inspiration. This was repeated one hundred times. The thorax was reopened and the clip removed from

the left mesobronchus. A dry glass cannula was inserted into the left posterior thoracic air sac and about one gramme of bismuth powder introduced. The thorax was again closed and the bird left for one hour during which time the expiratory air currents in the left posterior thoracic sac distributed the bismuth powder along the course of the air stream. The bird was then killed and stereoscopic skiagrams of the carcass taken (see Pl. I, fig. 2).

RESULTS

Examination by stereoscope of the photographs taken showed that on the right side the bismuth carbonate had been carried by inspiration from the trachea to the right mesobronchus and along the entobronchi into the abdominal and thoracic air sacs on the same side. A proportion of the powder was lying in the system of parabronchi or fine air capillaries on the right side, while there was much less evidence of the entry of powder into the interclavicular and cervical sacs. This may have been due to operative interference with the functioning of these sacs. In the left lung, which had been cut off from inspiration by closure of the mesobronchus, bismuth was seen in the posterior thoracic air sac and also penetrating the lung by the recurrent bronchi which lie around the ostium. There was no bismuth in the mesobronchus itself.

DISCUSSION

Consideration of the opposing theories of respiration in birds is made easier by examination of the following figures obtained by analysis of samples of gas withdrawn by needle from the air sacs at the end of inspiration and analysed in the portable Haldane apparatus (quoted by kind permission of Dr G. M. Wishart). The average findings were as follows: abdominal air sacs O_2 19 %, CO_2 2 %; posterior thoracic air sacs O_2 17.4 %, CO_2 3.4 %; anterior thoracic air sacs O_2 16.3 %, CO_2 3.2 %; interclavicular air sac O_2 14.6 %, CO_2 5 %; cervical air sac O_2 15.6 %, CO_2 3.2 %. Makowski's [1938] figures for gas analysis, expired air from the trachea O_2 13.5 %, CO_2 6.5 %; abdominal air sac O_2 18.3 %, CO_2 2 %, agree with the above.

These figures show that the air in the abdominal air sac at the end of inspiration approximates most nearly in composition to atmospheric air, with the posterior thoracic, anterior thoracic and interclavicular sacs coming next, in that order. Air therefore passes into the abdominal sac with but little alteration in the lung, while a variable proportion of gaseous exchange takes place during inspiration of the air into the other

4. The results of the technique described support the contention of Juillet [1912] that air enters the air sacs by the primary ostium during inspiration and leaves by the recurrent bronchi during expiration.

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air sacs. This degree of approximation to atmospheric air of the samples taken from these sacs may be in proportion to the mobility of the walls of the sacs and therefore to the speed and directness of the flow of air into them. Some gaseous interchange takes place during inspiration, most during expiration.

The above facts concerning the interchange of gases in the lung during inspiration and expiration are in agreement with the results given by the insufflation method described. This shows that air enters the sacs on inspiration by the main respiratory channels which are ill-adapted for gaseous exchange, and leaves the sacs during expiration by the lesser channels which are adapted for gaseous interchange. The final link in the chain of evidence, viz. the mechanism of opening and closing the ostia, is not clear. There is no histological evidence of a valvular mechanism, but such, though not evident, must be efficient. Variation in pressure in the air sacs during respiration, which is easily demonstrable by attaching a recording tambour to a hollow needle thrust into the sac, must result from external forces applied to the sac, thus supplying the motive force of respiration. This is shown by Makowski's [1938] demonstration of such variations in the abdominal air sac attached to a lung rendered atelectatic by being freed from the ribs behind and allowed to shrink. Such a fibrosed lung must remain passive during respiration, but the pressure variations in the sacs attached to it are similar to those in sacs attached to a normal lung.

Thus it is concluded that, in the fowl, air enters the air sacs during inspiration via the main bronchial channels as a result, in part at least, of the bellows-like action of the air sacs. Some gaseous exchange takes place during inspiration. On expiration the air is driven from the air sacs via the recurrent bronchi through the parabronchial air capillaries and gaseous exchange is completed.

SUMMARY

1. A brief synopsis of the anatomy of the lungs of the bird and the controversy with regard to the mechanism of respiration is given.
2. The technique of insufflating the lungs of the anaesthetized fowl with radio-opaque dust in order to secure a record of the direction of the air currents during inspiration in the one lung and expiration in the other lung is described.
3. The results of analysis of the contents of the air sacs indicate that the major proportion of gaseous interchange takes place during expiration.

When silver or platinum electrodes were placed directly in the vagina, the skin electrode was replaced by a thin silver or platinum wire inserted for a length of about 3 cm. under the skin of the upper abdomen or flank.

(2) The vaginal electrodes most often used consisted of Ag—AgCl as above, leading to glass tubes or No. 10 gum elastic catheters of 4–6 mm. bore, entirely filled with saline and closed at the inner end by a small wad of cotton-wool. From glass tubes this wad projected so as to give a possible contact area of about 6×4 mm. From catheters the wool was extended as a ring about 5 mm. wide, tied with thread round the catheter or catheters in use. In experiments where the vagina was distended with saline, no wool was used, and the "electrode surface" consisted of the oval orifice in the side of the catheter (about 7×9 mm.). The exact position of the vaginal electrode was usually determined by opening the abdomen after an experiment. The catheter orifice was usually about 85 mm. from the vulva and 35–65 mm. from the uterine apertures at the inner end of the vagina. The "contact area" of the electrode was thus in the middle third of the vagina. In a large rabbit the vagina may be over 150 mm. long. The inner third is usually anteverted, at least in advanced pregnancy.

The platinized glass electrode was a glass tube of 6 mm. external diameter, with the innermost 10 mm. platinized on the surface and in electrical contact with a platinum wire sealed through the glass.

All saline solution consisted of 0.9 % NaCl and 0.0042 % KCl in distilled water.

Potentiometers

The electrode wires led usually to a three-stage D.C. amplifier with push-pull input similar to that described by Matthews [1938], but with battery valves (H.L. 2 in the input stage) and an input resistance of 4 M Ω . This led to a robust insensitive galvanometer of period about $\frac{1}{10}$ sec. (H. Tinsley and Co.) and a recording camera. Alternatively a rotating switch with A.C. amplifier and cathode-ray oscillograph was used, in a method which it is hoped to describe shortly.

In the later records the unbalanced grid current and unbalanced tissue electrode voltage were kept too low to record any appreciable apparent voltage change for a change in input resistance of less than 200,000 Ω . As the resistance across the rabbit (including cotton-wool electrodes) was only about 660 Ω (at 10,000 cycles), it is safe to conclude that changes in biological resistance did not affect the observed potentials.

In all records shown a downward movement of the line indicates increasing negativity of the vaginal electrode.

Preparation of rabbits

The rabbits were anaesthetized by the intravenous injection of dial, 0.75 c.c./kg., aided by ether in experiments involving incisions. This gave prolonged quiescence without mechanical fixation. The rabbit was laid on its back on cotton-wool on a highly insulated shallow trough consisting of two boards sloping gently towards the centre line. Care was taken to see that no moist wool could touch surrounding metallic objects. This precaution is of great importance in slow potential measurements.

For window experiments an oval ebonite frame carrying a celluloid window 64×89 mm. was inserted between the flaps of skin, or skin and muscle, produced by a midline abdominal incision. A radiator lamp was adjusted about 30 cm. above the window to give rough control of the temperature and moisture below it. The insertion of this window seemed to have little effect on potentials recorded from substernal and vaginal electrodes.

Ergometrine was given by injection into an aural vein of a 0.1 % solution of the sulphate in water.

VAGINAL POTENTIALS IN RABBITS

By R. B. BOURDILLON

*From The National Institute for Medical Research, Hampstead, N.W. 3**(Received 19 July 1939)*

IN contrast to the great amount of work on electro-cardiography and encephalography, but little has been published on the slower potential changes related to visceral contractions in intact animals or men, although recent American work on ovulation potentials is of interest [Burr, Hill & Allen, 1935; Reboul, Davis & Friedgood, 1937]. The following studies in rabbits were made in the hope that these and similar potential changes in man and in animals may prove of diagnostic value, as well as of physiological interest.

If saline electrodes are placed on the skin of the abdomen, or one on the skin and one in the vagina, of an intact rabbit, a series of slow rhythmic potential changes can be observed under suitable circumstances. It is fairly easy to detect among these changes specific rhythms that occur simultaneously with contractions of the diaphragm, of the small intestine, the caecum, the uterus and the vagina. The present paper describes experiments on the "slow" potential waves associated with contractions of the vagina. I am not aware of any account of these potentials, although Rogers [1938] has described the much slower changes in vaginal potential associated with the oestrous cycle in the rat. Contractions of the intact vagina have been described for rabbits by Jastreboff [1884], Langley & Anderson [1895, p. 123], and for women by Kolbow [1938]. A thorough study of isolated rings of vaginal muscle by Dworzak [1938] is also of interest.

METHODS

Electrodes

(1) For the skin the usual electrode was a straight glass tube of 6-9 mm. bore held by a clamp so as to exert moderate pressure on the abdominal skin, which had been clipped or shaved. The glass tube was full of saline which led to Ag-AgCl electrodes of large surface area, prepared as described by Burr, Lane & Nims [1936], and mounted in a water bath.

associated with contraction of the muscle adjacent to the electrode.
 2) A more rapid positive swing, usually complete in 1-3 sec. This may occur at any part of the first element. Radiographic and visual evidence suggests that it is associated with bands of dilatation (inhibition) which

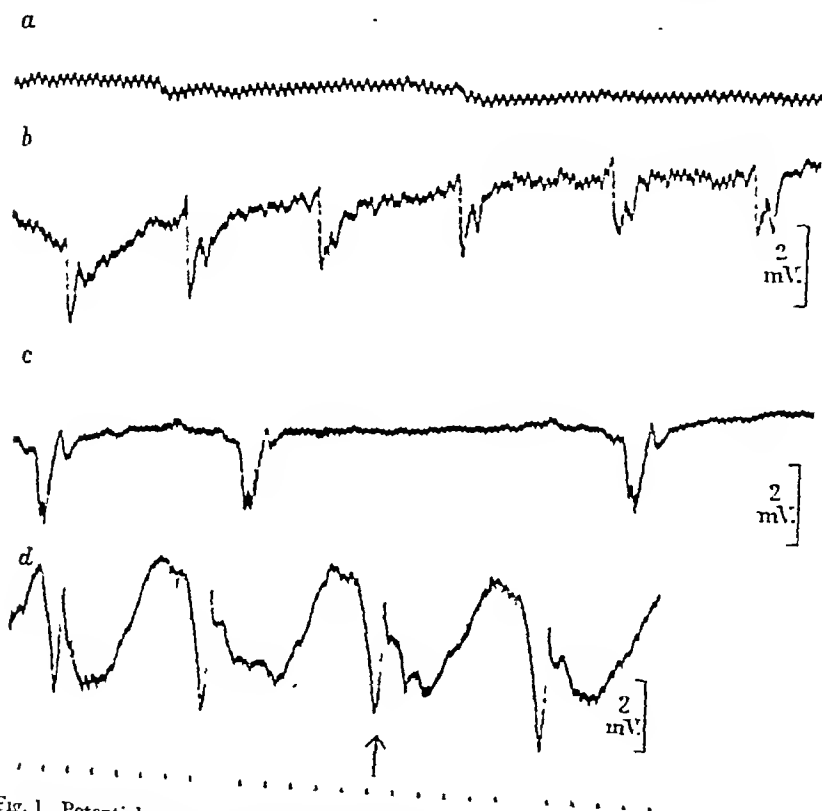


Fig. 1. Potential waves between vagina and abdominal skin of rabbit. *a*. At rest (showing respiratory wave only). *b*. Directly after aural injection of ergometrine. *c*. 10 min. after the injection. *d*. Similar waves with vagina distended with saline (and barium sulphate) 4-5 min. after ergometrine. The arrow signals the moment at which radiograph Fig. 2 *b* was taken. In this and later illustrations time marks are at 5 sec. intervals, and downward movement of the line indicates increased negativity of the vagina.

tend to appear on either side of a contraction wave, or sometimes in between two adjacent contraction waves (Pl. I, fig. 2 *b*). These two elements result in curves closely similar to those recorded by Orbeli & v. Brücke [1910] from the dog's ureter. These workers noted a negative potential of 0.3-3 mV. accompanying contraction, preceded, and usually followed

Dilatation of vagina with saline

In experiments involving this, saline (or barium sulphate and saline) was admitted to the vagina by a T-piece connected between the catheter and the silver chloride electrode. As soon as the desired volume (about 21 ml.) had entered, the T-branch was closed to prevent regurgitation through the catheter. The labia were tied round a short length of rubber tube surrounding the catheter so as to give a fairly watertight junction.

Time mark

In all illustrations the time marks are at 5 sec. intervals with every twelfth mark omitted. Curves read from left to right.

RESULTS

Vaginal potentials

In the normal fasting pregnant animal, with one non-metallic electrode resting in the middle portion of the vagina, and one held on the skin substernally, a fairly steady base line was obtained (Fig. 1a) with a potential difference not exceeding a few mV., and interrupted only by a respiratory wave due to contraction or movement of the diaphragm. This wave appears in most of the illustrations to this paper. The cardiac wave was sometimes visible, but is not obvious in most of the records owing to the nature of the recording system.

If the vagina was stimulated by the intravenous injection of ergometrine (0.3–0.75 mg./kg.) a series of potential waves was usually recorded as in Fig. 1b and c. The intervals between successive waves may be only 10 or 20 sec. at first, but later may increase to several minutes. After from 5 to 20 min. or more the waves cease. Similar waves can be obtained in susceptible pregnant animals by distending the vagina with saline, or in some cases by vigorous pressure with or movement of the electrodes. I have also obtained them after injection of adrenaline, but not after pituitrin. This agrees with the results of Dworzak [1938].

If ergometrine is given, these waves can be produced at all stages of the pregnancy cycle, and even in non-pregnant nulliparous animals, though in non-pregnant animals the waves have been less vigorous.

Shape of potential waves

The shape varies even in the different members of one series of waves, but the majority conform to a general type, which is easily recognizable, and appears to consist of two or possibly more elements. (1) A negative swing of the vagina reaching a maximum of from 1 to 6 mV. within 1 or 2 sec. from onset, and lasting from 3 to 10 sec. The recovery from this is usually slower than the onset. It is believed that this phase is

associated with contraction of the muscle adjacent to the electrode. (2) A more rapid positive swing, usually complete in 1-3 sec. This may occur at any part of the first element. Radiographic and visual evidence suggests that it is associated with bands of dilatation (inhibition) which

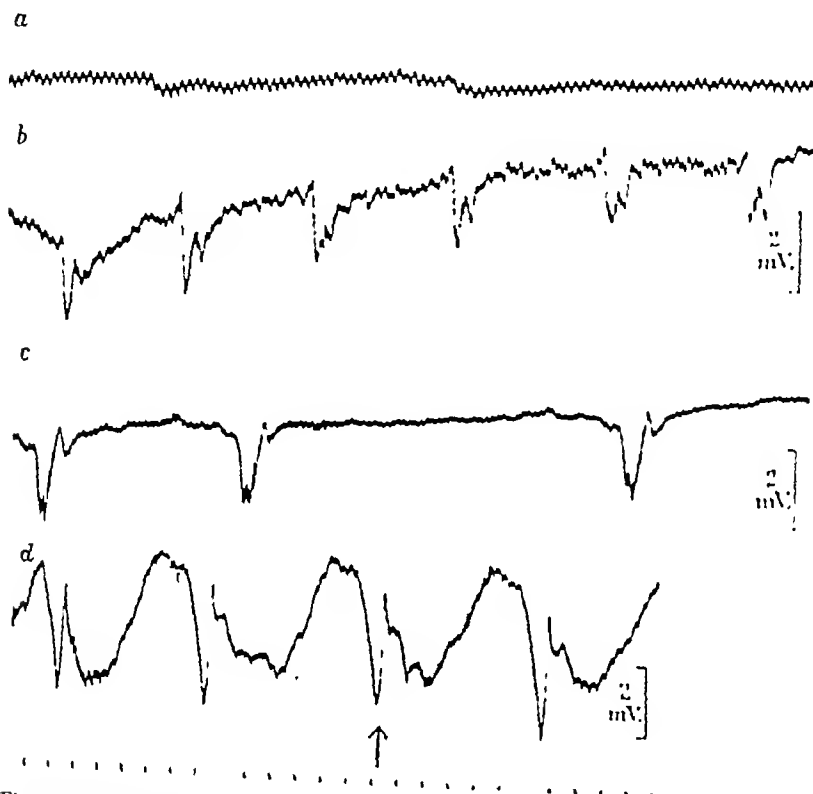


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by, smaller positive potentials which they attributed to nervous inhibition. (3) In the central portion of certain of the waves a series of relatively fast oscillations (period estimated at 0.05–0.9 sec.) can be seen. They are most conspicuous at *G* in Fig. 3, but can also be seen in Fig. 1 *b* and *c*. I have no evidence to show whether these are due to movement at the electrode, or represent action potentials of the type described by Bozler [1938], but I hope to study this point shortly. In the present work the recording system was only adapted for "slow" potentials, and fast changes such as the "initial potentials" of Rosenblueth, Leese & Lambert [1933] would, therefore, escape notice.

Proof of vaginal origin of potential waves

(1) Pl. I, fig. 2 *a*, shows a radiograph of a vagina slightly distended with saline containing barium sulphate in suspension, and radiographed approximately 3 min. after a dose of ergometrine (0.5 mg./kg.). The series of contractions and dilatations gives a good illustration of the vigorous reaction of the vagina to ergometrine. This photograph was taken shortly after recovery of the potential level from a "wave", and shows the catheter with its orifice surrounded by barium.

Pl. I, fig. 2 *b*, shows a radiograph taken with $\frac{1}{2}$ sec. exposure at the moment indicated by the arrow in Fig. 1 *d*, i.e. at the moment of a sharp upward swing (increasing positivity of vagina) during the early part of a slow potential wave. It is believed that the small bar of barium sulphate seen just across the catheter orifice represents a small dilatation coming between the contraction waves seen on either side, and that this is responsible for the brief positive swing seen on the record.

Pl. I, fig. 2 *c*, shows a radiograph taken at the moment of onset of maximum negativity in a potential wave some 15 min. after an ergometrine injection. It will be seen that the vagina has fewer contraction waves, but is firmly contracted at the catheter orifice.

(2) Visual evidence was also obtained by observation through an abdominal "window" of vaginas distended with saline and stimulated by ergometrine. In a full-term pregnant rabbit very vigorous contraction waves could be seen starting apparently near the uterine aperture and travelling slowly down the vagina.

Fig. 3 shows the potential changes associated with two such waves. The letters *F* and *H* signal moments when the uppermost part of the vagina was seen to contract, *G* and *J* the moments when the contraction waves were seen to reach the catheter orifice. In this experiment the catheter orifice had been surrounded with two rings of gutta-percha

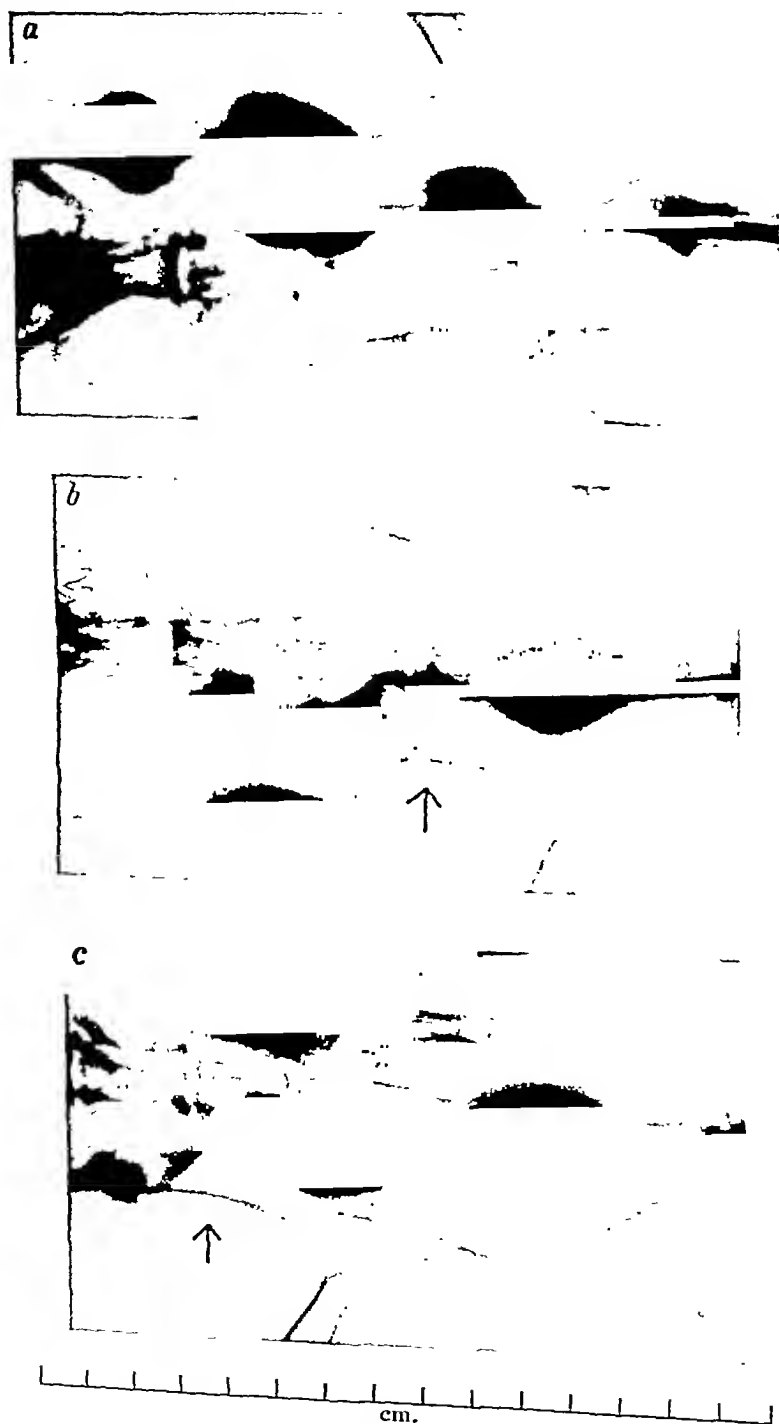


Fig. 2.

projecting by 5 mm. on two sides of the aperture, so as to prevent complete sealing of this during a contraction.

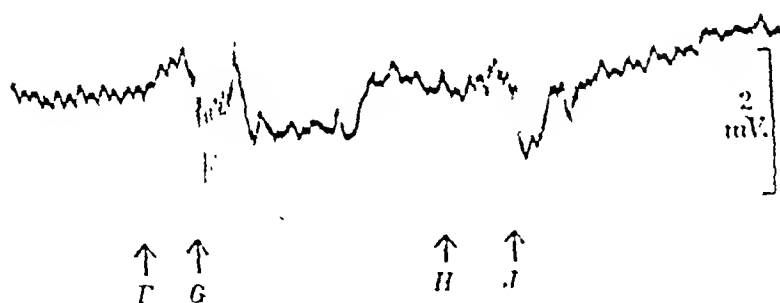


Fig. 3. Potential waves during contractions observed visually. Letters *G* and *J* signal the moments when contractions were seen to reach the catheter orifice.

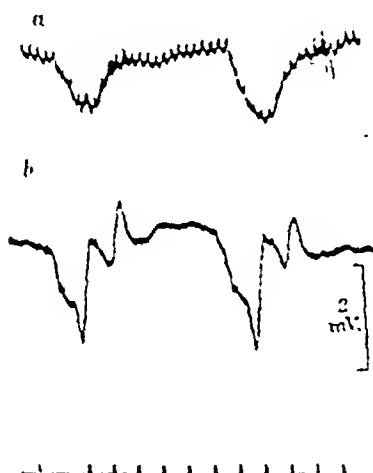


Fig. 4. Potential waves accompanying vaginal contractions. *a*. Recorded from vagina to substernal skin. *b*. From two vaginal electrodes, one 33 mm. ahead of the other. Downward movement indicates increasing negativity of the anterior vaginal electrode.

(3) Fig. 4 shows a series of waves initiated by ergometrine and recorded with one substernal electrode and one double electrode in the vagina. This double electrode consisted of two catheters tied side by side with the orifice of one 33 mm. ahead of the other, each closed with

cotton-wool. In Fig. 4 *a* the potential recorded was that between the substernal and the proximal vaginal electrodes. In *b* the potential between the two vaginal electrodes was recorded. This confirms the vaginal origin of the potential by showing, as would be expected for a contraction moving distally, an upward wave (i.e. a relative positivity of the anterior electrode) during the second half of the compound wave. The origin of the fall immediately preceding this positive wave is uncertain, but it fits well with the hypothesis that a wave of inhibition often closely precedes a contraction wave.

(4) Both visual and electrical observations showed that under the conditions of these experiments the uterine contractions and potentials were much more continuous and irregular than the vaginal waves here recorded, and that there was no danger of confusing the two.

Exact site of origin of potential waves

While I think the above evidence shows conclusively that the waves illustrated in Fig. 1 are associated with contractions of the vagina and not of any other organ, it is much more difficult to be certain how far they are true records of potential changes occurring in the muscle, and how far they are due to phenomena occurring at or near the electrode surface. Whereas with action potentials of voluntary muscle, or of nerve, the period is so short as to render the distinction of artefacts due to movement relatively easy, these "slow" potentials are of the same order of duration as the muscular movement itself, and therefore inferences as to their exact origin should only be made with the greatest caution.

Among the numerous possible causes of such potential changes the following have been considered seriously.

(1) That the potentials are due to summation of "action potentials" or to some similar process.

The chief evidence supporting this is: (*a*) The slower potentials obtained with a vaginal electrode have always indicated increased negativity of the vagina, and, although a short positive potential is frequently observed during the major wave, the appearance of this can readily be explained as due to a narrow bar of dilatation such as that seen in Pl. I, fig. 2*b*. (*b*) Similar types of wave are obtained with electrodes consisting of cotton-wool and saline, of saline in catheters with open aperture, and of platinized glass. (*c*) The potentials appear to coincide in frequency and duration with observed contractions of the vagina, and are much more regular than would be expected if they were caused by movement at the electrode.

(2) That the potentials are artefacts due to friction between the moving muscle and the vaginal electrode.

This is clearly disproved by the typical potential waves obtained, as in Fig. 1d, from a closed vagina partially distended with saline, and containing as electrode an open-ended catheter filled with saline. Although true friction is thus excluded, this type of electrode might show potentials during movement, due to "streaming" of electrolyte, especially if aided by unsuspected changes in temperature or concentration near the catheter orifice. However, if such effects were conspicuous, it is so unlikely that they would give potentials of sign, amplitude and duration, closely resembling those obtained with solid electrodes, that this explanation appears improbable.

(3) That the potentials are due to the successive contact with the electrode of different portions of vaginal epithelium at differing steady contact potentials.

This possibility is difficult to exclude. It has been tested in several ways as follows:

(a) *Use of suction electrode in vagina.* At the suggestion of Dr G. L. Brown tests were made with a vaginal electrode fixed by suction to an exact spot on the vaginal wall. The electrode ended in a piece of rubber tubing full of saline and with a slanting open end of 4.5×8 mm. aperture, fixed by a suction equal to 36 in. head of saline. The application of this suction apparently irritated the vaginal wall, as it was promptly followed by a series of potential waves each of about 6 sec. duration and 5 mV. amplitude. After injection of ergometrine a further series of similar potential waves was recorded. These waves were abnormal in shape, probably owing to damage to the vaginal epithelium in the area exposed to suction. Post-mortem examination showed considerable congestion and roughening of this area, so that normal potentials could not be expected. However, the record does show conclusively that large "slow" potentials can be observed in the absence of any major translatory motion.

(b) *Manual movement of vaginal electrode.* The potentials resulting from manual movement of cottonwool-saline-catheter electrodes up and down the vagina are very variable. On some occasions to and fro movements of 2.5 cm. will only cause potential changes of 0.2-0.3 mV. More usually the changes are of the order of 1 mV., and after prolonged experiments with ergometrine such manual movements have caused apparent potential differences of 2-4 mV. between portions of the vagina 2.5 cm. apart. The admission of a little fresh saline to the vagina does

not greatly reduce these effects. Similar potentials have been observed 15 hr. after death by air injection or by KCN injection: also after instilling 10 c.c. of 2 % cocaine and, in another case, 20 c.c. of chloroform into the vagina.

These potentials may be related to the prompt muscular relaxation which occurs after one or two rubbing movements in a vagina that has been at rest and shows the usual mild contraction gripping the catheter. It seems, however, more probable that these potentials are due to differences in the ionic content of the vaginal secretion at different spots, either existing normally, or excited by local pressure of the catheter as well as by friction.

It is impossible to say that these potentials have no effect on the records shown in Fig. 1, etc., but there are many reasons (such as their low value for a small movement and the irregularity of sign shown on different occasions) for thinking that they do not play a major part.

(c) *Tests with metallic electrodes.* To prove that the potentials observed were not dependent on any peculiar property of saline or cotton-wool saline electrodes, a few tests were made with metallic electrodes used without saline. Fig. 5 shows the potentials recorded between a platinized glass vaginal electrode and a platinum wire in the flank: (a) shortly before, (b) 6 min. after the injection of ergometrine (0.62 mg./kg.). These platinum electrodes showed a resting P.D. of 120 mV., which was fairly steady before the ergometrine, but rose rapidly after it to 172 mV., presumably from some alteration in the vaginal secretion caused by the ergometrine. The drift is obvious in the record, but if allowance is made for its effect, the potential waves due to vaginal contractions are seen to be very similar to those observed with saline electrodes.

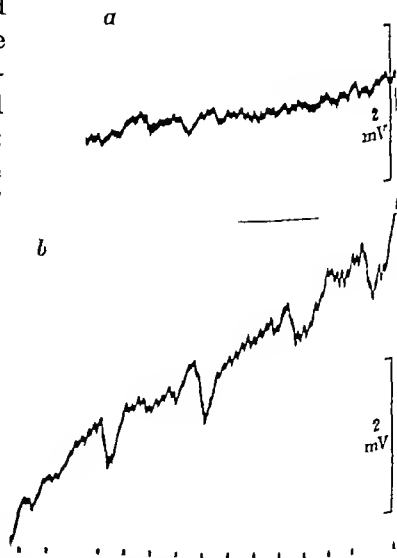


Fig. 5. Potentials between platinum electrodes. a. Before ergometrine. b. 6 min after it.

Attempts were made to record potential waves from a hook of silver wire inserted from the abdomen

into the vaginal wall. The results obtained were irregular and unsatisfactory, owing apparently to local injury to or tension on the vaginal wall affecting its contractility at the electrode site. Dworzak [1938] has commented on the need for gentleness in handling the vagina if satisfactory contractions are to be observed.

(c) *Use of two electrodes on abdominal skin.* While the above tests may be regarded as suggesting that movement near the electrode is not the major cause of the vaginal potentials observed after ergometrine, the results are inconclusive, owing to the active responses of the vagina to friction. It is therefore worth considering the following evidence obtained without the use of any electrode in the vagina.

Transmission of "slow" potentials through the abdominal wall

That slow potential waves produced in the abdomen can be recorded by electrodes placed on the skin is shown in Fig. 6 and Table 1. This shows the potentials recorded from two glass-tube saline electrodes placed

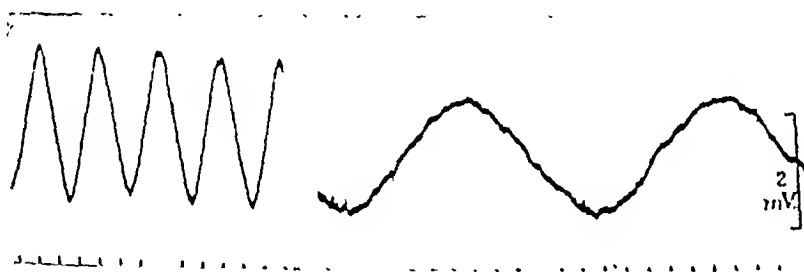


Fig. 6. Low frequency sine waves recorded after transmission through the abdominal wall. Periods 15.0 and 59 sec. Input mV. (in abdomen) 7.1 and 8.1 (peak). Recorded mV. (on skin) 1.23 and 0.98 (peak).

on the abdominal skin of a rabbit 65 mm. apart in the midline about half-way between symphysis and xiphisternum. A liquid low-frequency generator was used to produce a sine wave of frequencies from 1 c./sec. down to 1.60 c./sec. This was applied to two silver electrodes ($38 \times 10 \times 0.35$ mm. wrapped in five layers of thin linen and sealed to wires leading through glass tubes) which were inserted through small holes in the flank so as to lie on the abdominal viscera below the anterior wall and directly under the recording electrodes. The applied peak current and peak voltage were measured on d.c. instruments, and in rabbit 59 the input voltage was also recorded with the camera used for the skin electrodes, so as to get greater accuracy.

TABLE I. Transmission through abdominal wall of potentials of very low frequency (sine waves). The figures give "peak" currents and voltage, not R.M.S. values.

Rabbit no.	Current applied internally			Voltage recorded from skin	
	Period sec.	μ A.	Voltage mV.	mV.	% of input voltage
59	0.95	—	3.84	1.13	29.5
	1.83	—	4.59	1.25	27.1
	3.7	—	6.1	1.5	25
	7.5	15.4	6.6	1.35	20.5
	15.0	14.6	7.1	1.23	17.3
	29.3	12.9	7.55	1.07	15
	59.0	5.1	8.1	0.98	12
58	9.1	10.2	4.0	1.0	25
	14.5	9.5	4.3	0.92	21.4
	18	9.5	5.0	0.9	18
	59	8.7	5.0	0.65	13
41	10.4	5.8	3.0	0.65	22
	10.4	16	9.0	1.9	21
	50	12	12	1.5	12

Owing to the limitations of the instruments available the values obtained are only approximate, but Table I shows that about 12 % of the applied voltage is transmitted at a period of 60 sec. and that the transmission increases to 29 % at a period of 1 sec. The values obtained for three different rabbits agree better than would be expected in view of probable variations in the thickness of the abdominal wall.

In rabbit 41 at a period of 10 sec. a rise of current from 6 to 16 μ A. causes little fall in the percentage transmitted through the skin.

The fall of transmission with decreasing frequency is presumably due to polarization at or near the peritoneum caused by a flow of current along the voluntary muscles of the abdominal wall.

Recording of vaginal potentials through the abdominal wall

In two cases we have obtained recurrent potential waves of the "vaginal type" between two electrodes (glass tubes with saline) held on the abdominal skin.

Fig. 7 shows the record obtained with two electrodes pressed on to the abdominal skin of a full-term pregnant rabbit, one substernal, and the other about 2 in. above the symphysis, between the bulges due to the loaded uterine horns. It is believed that this record shows vaginal potentials transmitted through the abdominal wall, since they have the characteristic shape, frequency and number, of vaginal waves expected after ergometrine. Their amplitude is about 0.8 mV., which is about one-third that of the waves recorded in Fig. 1 *b* and one-fifth of that in Fig. 1 *d*. This fits tolerably with the transmissions given in Table I.

As the rabbit's vagina is usually anteverted at the uterine end, its upper portion can come close to the anterior wall of the abdomen, especially when this is pressed down by an electrode in the midline. However, I have sometimes failed to record such waves through the skin, probably owing to intervention of portions of the uterus between vagina and skin.



Fig. 7. Potentials recorded with both electrodes on the abdominal skin 2 min. after ergometrine. Believed to be vaginal in origin except for small irregularities transmitted from the uterus.

Possible effect of visceral movements on superficial electrodes

The following "balloon" tests were made to determine how far potentials recorded from the abdominal skin could be produced by mechanical movements of the viscera.

A thin cylindrical rubber balloon about 25×120 mm. was inserted through a small hole in the flank so as to lie transversely just under the abdominal wall with its axis, about 1 in. distal to a substernal electrode. With a second electrode in the vagina, potentials were recorded after (1) inflating the balloon enough to cause a slight protuberance of the abdominal wall, (2) further inflation to form a very large protuberance pressing the skin up immediately to the side of the electrode tube, (3) suddenly releasing the pressure.

Fig. 8 *a* and *b* shows the effects of these operations in two different rabbits. The first shows only a small effect (*PQR*). The second a change of 1.3 mV. after the second inflation (*TUV*). Both types have been observed repeatedly. It is possible that the potential change sometimes following the sudden release of pressure is due to stimulation of a viscus causing an actual internal potential change, but we have no conclusive evidence for this. Similar tests with inflation and deflation limited in rate to about 60 c.c. in 4 sec., with the balloon directly under the electrode, usually gave no obvious potential changes in the record.

Fig. 8 *d* from *K* to *L* shows the small effects produced by scraping the anterior abdominal wall directly under the skin electrode with a glass rod with light pressure. *M* shows the effect produced by moving a mildly inflated balloon to and fro under the electrode.

Fig. 8 *c* shows for comparison a potential wave believed to be due to the "natural" contractions of a flatulent bulky caecum, obtained with one substernal and one vaginal electrode. The caecal movements were visible through the abdominal wall, but appeared less vigorous than

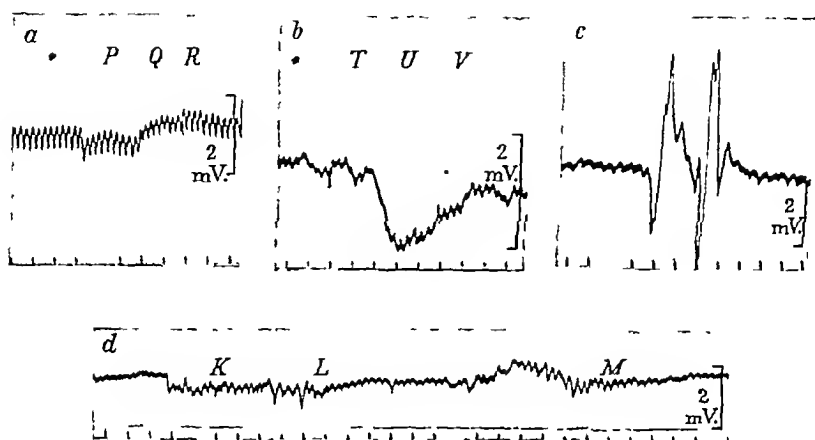


Fig. 8. *a* and *b* show the effects of rapid inflation and deflation of a balloon in the abdomen close to the substernal electrode in two different rabbits. *P* and *T* signal mild inflation; *Q* and *U* come after severe inflation; *R* and *V* after sudden deflation. *c* shows for comparison a wave due to natural contraction of a flatulent caecum. *d* shows effects of movements of glass rod and balloon as described in text.

the balloon movements recorded in Fig. 8 *a* and *b*. The contrast is striking, and makes it difficult to avoid believing that the potentials in Fig. 8 *c* are genuine muscle potentials.

These tests show that considerable movement just under the abdominal wall may cause only trifling potential changes at superficial electrodes, or may cause serious changes of 1 or 2 mV. It seems probable that the serious changes are due to visceral contraction, stimulated by the sudden change of pressure, but, as with the other movement tests, the evidence is not conclusive.

Taken in conjunction Figs. 6–8 afford good grounds for thinking that the potential waves observed with vaginal contractions represent genuine muscle potentials rather than artefacts due to movement at the electrode.

For in Fig. 7 the vaginal movements must have been much smaller than those of the balloon, etc., in Fig. 8, yet they produced characteristic waves.

CONCLUSIONS AS TO THE ORIGIN OF POTENTIALS

It seems evident that the vagina is so sensitive to friction or pressure that it is difficult to be certain that secondary effects produced by movement play no part in the potential waves here recorded. It is even possible that the brief positive swing seen in many records is due to a dilatation wave itself caused by friction or pressure of the moving vagina on the electrode. But, with these reservations, I think the balance of evidence favours the hypothesis that the potentials recorded do in their main outlines represent genuine activity potentials in the vaginal wall, probably chiefly arising in the muscle layers. Further, these slow potentials can be of practical value as a means of studying vaginal physiology. Whatever their precise cause they correspond closely to vaginal contractions in frequency and in duration, and probably in form and amplitude, and they may thus afford a tool for research into involuntary muscle comparable in value to the action potentials of voluntary or cardiac muscle.

SUMMARY

1. Vaginal potential waves of duration 5-12 sec. each, and amplitude 1-6 mV., have been recorded in both pregnant and non-pregnant rabbits.
2. Radiographs as well as visual observations show that these are associated with vigorous contractions of the vagina, the active portion becoming negative while contracted. These contractions can be produced by intravenous ergometrine or adrenaline, and sometimes by distending the vagina with saline.
3. The origin of the potential waves is discussed, and a number of experiments described which tend to show that the observed potentials represent genuine potentials produced in the vaginal wall rather than mere artefacts due to movement at the electrodes.
4. Although their exact origin is still open to doubt, it is safe to say that these potentials can form a valuable tool in studying the physiology and pharmacology of the vagina.

I wish to express my thanks to Sir Henry Dale and Dr G. L. Brown for advice on this problem, and to Dr E. Schuster for the design and construction of the camera which played an essential part in this work.

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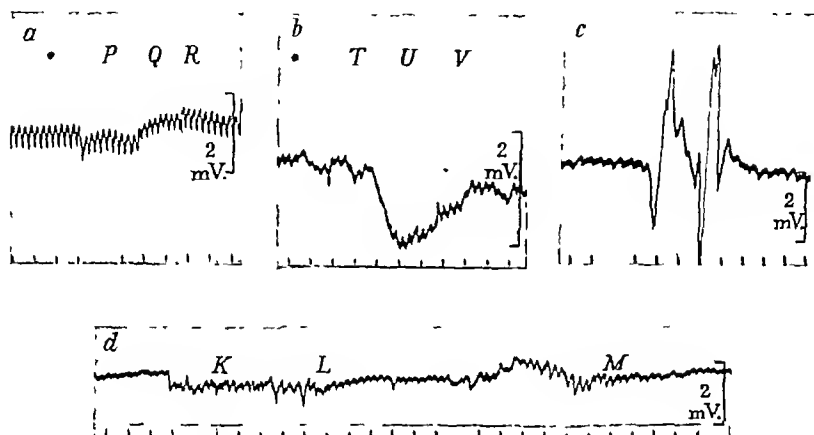


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EXPLANATION OF PLATE I

Fig. 2. Radiographs of the vagina of a 4 kg. rabbit distended with saline and barium sulphate. An arrow points to the catheter orifice in *b* and *c*. *a*. About 3 min. after injection of ergometrine, showing three major contractions. *b*. At the moment signalled in Fig. 1 *d*. Note the narrow bar of barium sulphate (dilatation or inhibition) directly opposite the catheter orifice, also the closely adjacent contraction waves. *c*. At a later phase, taken at a moment of maximum negativity in a potential wave. Note the catheter orifice just clear of the barium, indicating full contraction at this spot.

IMPULSES IN THE PYRAMIDAL TRACT

BY E. D. ADRIAN AND G. MORUZZI¹*From the Physiological Laboratory, Cambridge**(Received 28 July 1939)*

ALTHOUGH it is easy to demonstrate the electrical activity of the brain we are still some way from understanding the full meaning of our records. This is mainly due to the complex structure in which the potential changes occur, though it is true that to simplify the conditions various means have been devised for recording from restricted areas, e.g. by micro-electrodes, multiple leads, etc. In the present work the problem has been approached from a different angle, for we have been chiefly concerned not with the potential waves in the cortex but with the impulses sent down the axons of cortical cells. These have been recorded by leading from the fibres of the pyramidal tract in the medulla: with suitable arrangements it is possible not only to study the composite discharge in many fibres of the tract, but to distinguish the impulses in single conducting units. We have, therefore, a method of determining the activity of the Betz cells in different conditions and we can compare the pyramidal fibre discharge with the potential waves in the motor area. The method has led to several unexpected but illuminating results. In particular it has been found that in the anaesthetized animal there is usually a continued pyramidal discharge which is infra-liminal for the motor nerve cells of the spinal cord, that the cerebral neurones can be made to discharge at extremely high frequencies with certain forms of stimulation, and that such discharges are to be found whenever there is a widespread excitation of epileptiform type.

METHOD

The possibilities of the method were discovered accidentally. It was known that axon potentials could be detected in the white matter of the cortex by an insulated wire electrode [Adrian & Matthews, 1934].

¹ Fellow of the Rockefeller Foundation.

Barron & Matthews [1935] had been able to record impulses in single fibres of the spinal ascending tracts and Gesell, Bricker & Magee [1936] had published similar records from the neurones in the medulla concerned with respiration. But although the brain of an anaesthetized animal is by no means inactive electrically, it seemed unlikely that it would give efferent discharges worth recording, and in any case unlikely that a way would be found for recording from single units.

In an experiment on the sensory nuclei, however, it was noticed that a wire electrode leading from certain restricted areas of the medulla picked up an axon discharge with fluctuations of 0.05 mV. or more occurring every few seconds at a frequency of 7-10 per sec. The animal, a cat, was anaesthetized with dial, and in cats under this anaesthetic the potential waves in the cerebral cortex have the same characteristic arrangement. As soon as this fact was called to mind a series of tests made it increasingly clear that the discharge in the medulla was derived from the pyramidal tract and came from the motor cortex. It could be recorded only from the region of the tract or of the decussating fibres, and it was abolished by destroying the motor cortex of the appropriate side and modified by stimulating it, whereas injury or stimulation elsewhere had no specific effect. In fact the anaesthetic instead of rendering the Betz cells inactive had given their discharge a characteristic pattern which made it easy to recognize.

The other essential step was to find a method for recording the impulses in single fibres, or rather conducting units, of the tract, and this again was easier than we had expected. To obtain such records the exposed end of the wire electrode must be brought close to one active fibre or group of fibres acting in unison and there must be no others near enough to produce comparable potential changes. In the pyramidal tract itself the fibres are so closely packed that this condition cannot be realized, but there is a much greater separation when the decussation is reached. Here the tract splits into small bundles which run through the grey matter to the opposite side, and in this region, with a very fine electrode, it is often possible to find a position which gives large action potentials alike in size and arranged in a definite series. Smaller potentials with a different arrangement may be present in the background, but in favourable cases there is little doubt that the large spikes are due to a structure which behaves as a single conducting unit.

The details of the method have been varied to suit particular experiments; its general nature is summarized in the diagram in Fig. 1. The brain figured in the diagram is that of a cat with the motor area far

forward. The method has been used successfully on monkeys in several experiments. A few made on rabbits have confirmed the main results, but there have been many failures owing to the smallness and limited distribution of the pyramidal tract.



Fig. 1. Method of recording from the motor cortex (electrode A), from the fibres of the pyramidal decussation (electrode B), and from the subcortical fibres (electrode C). An indifferent electrode leads from the scalp.

Detailed technique

Anaesthetic. All the animals have been under a general anaesthetic throughout the observations. In the earlier experiments dial was always used, since it gave the characteristic grouping of impulses in the pyramidal fibres and so made it easy to find the best regions to explore with the electrode. Later on, experiments were made with chloroform and ether (c.e.), ether alone, chloralose and magnesium sulphate, since these give a different pattern of cortical and pyramidal activity. When it was desirable to change from the one type of anaesthetic to the other during the experiment we used sodium evipan instead of dial. Evipan agrees with the other barbiturates in giving groups of potential waves at 7-10 per sec. in the cortex, but it produces a much shorter period of anaesthesia. It was possible, therefore, to locate the pyramidal fibres under evipan and then to change over to ether for the rest of the experiment.

Operation. As a rule the operation involved the exposure of the dorsal surface of the medulla and the exposure of one or both motor areas of the cortex. The dura was left intact over the cortex as long as possible, for we found that opening it often reduced the activity of the motor area, owing perhaps to the distortion or compression of vessels by the weight of the brain pressing on the unsupported frontal regions. Sometimes another opening was made farther back to expose the lateral surface of the cerebral hemisphere.

Electrodes. In the earlier experiments the head was fixed and the wire electrode was carried by a horizontal rod clamped to a post on the

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to eliminate slower changes. The potentials were photographed on bromide paper moving at speeds up to 40 cm./sec. and were also reproduced by a loud-speaker. Most of the preliminary exploration in each experiment was done with the loud-speaker alone.

Electrical stimulation. For stimulating the motor cortex electrically we have used either single condenser discharges or repeated break shocks from an induction coil with a rotating contact breaker in the primary. The make shocks were short-circuited by a different set of contacts on the contact breaker. The strength of the shocks was controlled by a resistance in the primary. The frequency could be set to different values up to 70 per sec. and the duration of each shock could be altered by condensers interposed in the secondary leads. No part of the apparatus was earthed as this is liable to increase the stimulus artefact in the record.

RESULTS

PART I. THE ACTIVITY OF THE PYRAMIDAL TRACT AND MOTOR CORTX

(a) *Spontaneous activity in dial anaesthesia*

Typical records of the pyramidal tract discharge in a cat under dial (0.5 c.c./kg.) are given in Fig. 3. They were made with an electrode arranged to show the summed activity of many fibres, and in both of them

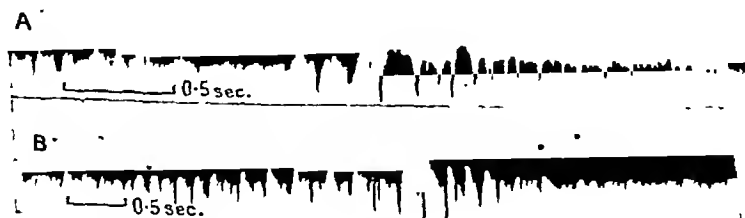


Fig. 3. A. Pyramidal tract discharge in a cat under dial, showing one of the active periods with groups of potential waves at 7-10 per sec. B. Ditto. Another animal. All records read from left to right.

this appears as a group of potential waves repeated every few seconds. During the group the frequency is 7-10 per sec.: between the groups the rhythm is slower, or the waves may be absent with small rapid excursions to show that the impulses have not ceased entirely though they are no longer discharged in synchronized volleys.

The regions which give this characteristic response correspond with the course of the pyramidal fibres. Thus if the motor cortex on one side

animal board. A precise movement of the electrode could be made by screw adjustments. With this arrangement we could map out the distribution of the pyramidal fibres (or rather of the characteristic discharge), for the position of the end of the wire could be gauged accurately in relation to the surface of the medulla. Later on the elaborate electrode stand was discarded in favour of a holder fixed to the skull. A small vulcanite support was fastened to the dorsal surface of the skull with sealing-wax, and this carried one or two short metal arms ending in a ball and socket joint by which the wire could be adjusted (Fig. 2). This arrangement was better for recording single unit discharges, for once the right adjustment of the electrode had been found there was no danger that it would be lost owing to slight movements of the electrode stand relative to the head.

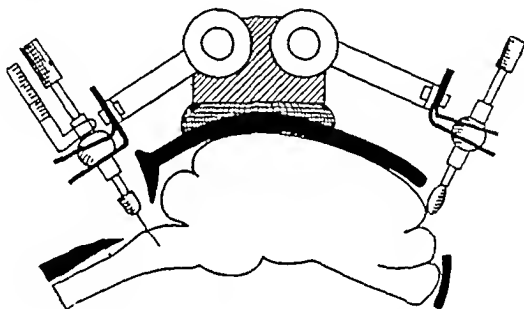


Fig. 2. Arrangement of electrodes for motor cortex and medulla.

The wire electrode was of silver, insulated with enamel except at the tip; the indifferent electrode was also of silver and was attached to the scalp. For general exploration we used a relatively thick wire (No. 38 or 40 s.w.g.), since this could be thrust into the nervous tissues more easily. For single unit recording, following the advice of Dr Matthews we found that the best results were given by very fine wire (No. 44 and No. 46 s.w.g., the latter having a diameter, including the enamel, of 40μ). For leading from the surface of the cortex we used an electrode of silver wire coated with silver chloride and ending in a moist thread or small tuft of cotton-wool. The same holder could be adapted to carry bipolar stimulating or recording electrodes.

Electrical recording. The potential changes in the medulla and cortex were recorded by two Matthews oscillographs, driven by amplifiers in which a balanced input stage could be used [Matthews, 1934]. In recording the axon potentials small coupling condensers were often used

was picked up the circuit from 4-6 V. battery was passed through the wire for 10-20 sec., the indifferent electrode being connected to the negative pole. The medulla was afterwards fixed in formalin, then sectioned and stained with ferrocyanide to show the position of the iron deposit. This always coincided with the bundles of decussating pyramidal fibres or with the uncrossed tract (cf. Fig. 4 B).

Motor cortex and pyramidal tract. The most striking confirmation of the nature of the discharge is given by simultaneous records from the medulla and from the motor area of the cerebrum. It is true that under

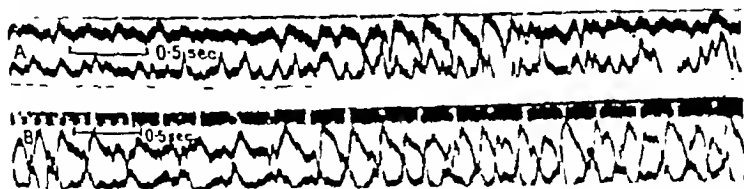


Fig. 5. Records from cerebral cortex and pyramidal tract. Cat under dial. A. Record from pyramidal tract (above) and striate area (below) showing lack of agreement in waves. B. Record from pyramidal tract (above) and motor area (sigmoid gyrus) below. Agreement during the group of large waves.

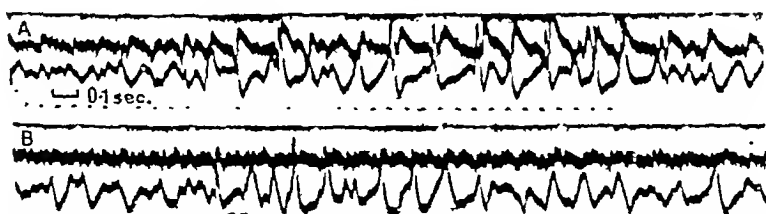


Fig. 6. Records from medulla (above) and motor cortex (below). Cat under dial. A. Medullary electrode in pyramidal decussation. B. Medullary electrode shifted 1.5 mm. deeper.

dial the potential waves from almost any part of the cortex would give the characteristic rhythm with groups at 7-10 per sec. and pauses between, but it is only the waves from the motor area which show a precise agreement with the medullary discharge. This is seen in Fig. 5 A and B. Fig. 5 A shows the lack of detailed agreement between the waves from the striate area of the cortex and those from the medulla. Fig. 5 B shows the very close correspondence when the cortical record is taken from the motor area (sigmoid gyrus). An equally close agreement is shown in Fig. 6 A (another preparation), but a slight shift in the position of the medullary electrode is enough to abolish the waves in the record from

has been destroyed or made inactive the discharge can be obtained from the regions shown in Fig. 4 A. On the side with the motor cortex intact it is to be found deep in the medulla and close to the midline; on the other side farther from the midline and more superficially, i.e. 1-3 mm. below the dorsal surface. The deep, ipsilateral response can be found

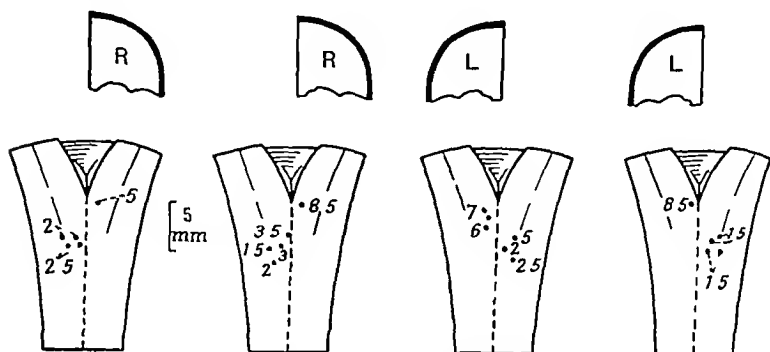


Fig. 4A. Position of regions giving typical pyramidal response in different cats. The side on which the motor cortex is intact is shown above each diagram of the brain stem. The figures opposite each dot show the depth from the dorsal surface (in mm. at which the response was found.

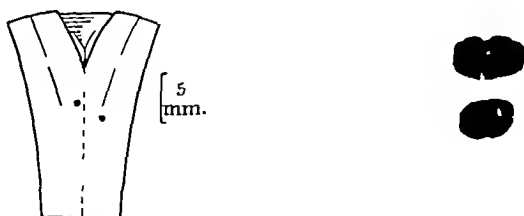


Fig. 4B. Sections of a brain stem with position of electrode marked by Hess's method (see text). Right motor cortex destroyed. In the upper section the stained area is in the uncrossed pyramidal tract, in the lower it is in the decussation. Diagram shows level of section.

above a level about 4 mm. caudal to the tip of the fourth ventricle: the crossed response comes from a region about 3-6 mm. caudal to the tip of the fourth ventricle. The distribution is obviously that of the uncrossed pyramidal tract and of its decussating fibres. This identification has been confirmed in several experiments by the method introduced by Hess [1932] for marking the position of an electrode in the brain. An enamelled steel wire was used instead of one of silver, and when the typical response

The direction of the potential changes shows that the indifferent electrode becomes initially negative to that in the medulla. This would follow from the fact that the indifferent electrode leads in effect from the whole length of the pyramidal axons: a more rapid recording system would probably show a triphasic variation corresponding to each impulse.¹

In most of our records the waves seem to be due entirely to fluctuations in the total number of impulses from moment to moment, for when the electrode is suitably placed for recording large impulse potentials there are no slower changes of appreciable size. It is true, however, that the electrode system cannot be made non-polarizable and might not show more gradual effects. These are occasionally present in addition to the impulse potentials, but we cannot say whether they are due to impulse discharges taking place at a distance from the electrode, or to some other kind of electrical activity (e.g. electrotonic spread from the Betz cells).

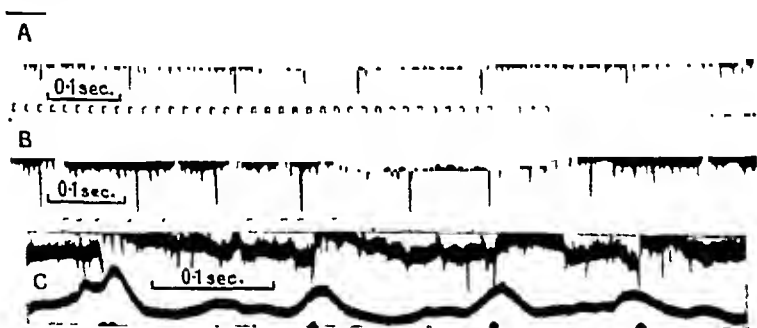


Fig. 8. "Single unit" discharges during one of the active periods. Three preparations. In C the lower tracing is from the motor cortex. The black dots mark the single unit potentials.

When the electrode has been arranged to lead from the decussating fibres a very slight shift in its position will often make one series of impulses stand out above all the rest, and if the series has a moderately regular arrangement and a uniform size it is fair to assume that it comes from a single unit in the cortex. The unit might be either a single neurone or a group of neurones linked so closely in the cortex that they are always in action together. It will be seen later (p. 183) that some of the units are certainly groups rather than single neurones. Typical single unit discharges are shown in Fig. 8. They are what might have been expected from the multi-unit records, for every few seconds the frequency rises to 10 per sec. with a slower and less regular discharge in the intervals. Simultaneous records from the motor cortex show that the group of impulses at 10 per sec. corresponds with the group of potential waves in

¹ Since this was in proof Lorente de N6 [1939] has published a detailed treatment of the conditions determining potential changes at buried electrodes.

the medulla (Fig. 6 B). The lack of agreement in Figs. 5 A and 6 B makes it clear that the agreement in the other records is not due to electrical spread of any kind.

To secure the best correspondence, the cortical and the medullary electrodes must be placed so as to record as far as possible the summed activity of the whole motor area and of the whole pyramidal tract. If the leads are restricted, that on the cortex will be derived mainly from a particular region of the motor area and that in the medulla will not necessarily come from the corresponding part of the pyramidal tract. The agreement will then be evident during each group of large cortical waves when the activity is more or less synchronous throughout the motor area; but in the intervening periods the pyramidal record may show waves at a lower frequency whilst the cortex seems inactive or vice versa.

To anticipate it may be said now that the results obtained in dial anaesthesia do not stand alone. Whatever the anaesthetic we have found no condition in which the discharge in the pyramidal tract is not in reasonable agreement with the potential waves in the motor area. But the pyramidal discharge is not always to be found, and there are certain states of the cortex in which surface potential waves can still be recorded from the motor area, although there is no corresponding discharge in the medulla. The conditions in which this may occur are dealt with in a later section.

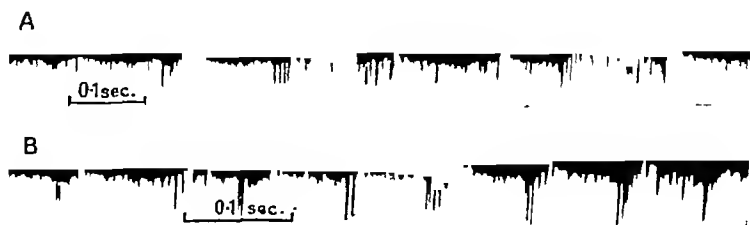


Fig. 7. Records from the pyramidal decussation with a fine wire electrode to show individual action potentials. A. Cat under dial. Record during one of the groups of impulses at 10 per sec. B. Ditto. Another animal.

Impulses in single units. With a fine wire electrode in the decussation it is easy to find positions in which the individual impulses can be seen, grouped together into brief outbursts corresponding to the waves and scattered irregularly during the quiet intervals (Fig. 7).

The other is that the effect is due in some way to injury of the pyramidal fibres by the wire electrode. These explanations will be considered in a later section and for the present the cause of the double and triple impulses must be left undecided.

(b) Spontaneous activity in chloroform and ether anaesthesia

It is well recognized that under chloroform and ether or ether alone the potential waves in the cerebral cortex present an entirely different appearance from those occurring under dial [Derbyshire, Rempie, Forbes & Lambert, 1936]. Instead of groups of abrupt, isolated waves at 7-10 per sec. there are small, regular oscillations at 40-60 per sec. often



Fig. 10. Record from cortex (A) and pyramidal decussation (B) in a cat under chloroform and ether, showing regular waves at 50 per sec.

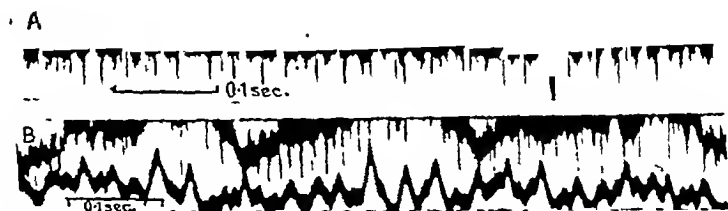


Fig. 11. Pyramidal discharge in ether anaesthesia. A. Record from several units. B. Another animal, simultaneous record from decussation (above) and motor cortex (below).

superimposed on slower movements of the base line [cf. Adrian & Matthews, 1934]. The rapid oscillation is often very close to 50 per sec. and regular enough to arouse the suspicion that it is an artefact from the A.C. mains; but in fact the regularity varies considerably, the frequency falls when the anaesthesia is very deep and the waves do not occur synchronously in different parts of the cortex.

The same 40-60 per sec. rhythm appears in the pyramidal tract. In Fig. 10, for instance, the first half of the record was made from the motor cortex and the second half, immediately after, from the tract in the medulla. Fig. 11 B gives simultaneous records from the cortex and tract in another experiment. With deeper anaesthesia the cortical waves and

the cortex (Figs. 8 C, 12 B). In the intervals between the groups the correspondence is not so well marked.

Both the single unit and the multi-unit records agree, therefore, with the view that the larger potential waves of the cortex represent a synchronous activity of deep and superficial layers, with a corresponding impulse discharge down the axons of the pyramidal tract. In an animal under dial this discharge produces no movement and no appreciable change in the tone of the muscles. It is therefore subliminal for the ventral horn cells of the spinal cord.

Multiple outbursts. We had not expected to find a motor discharge from the cortex in deep anaesthesia, but granted that the discharge occurs its relation to the cortical potentials is not surprising. There is, however, one feature of the single unit records which is not found in more familiar types of motor and sensory discharge. This is the occurrence in some records of two or more impulses very close together in place of a single



Fig. 9. Single unit discharges showing multiple outbursts (2-3 impulses in each).
Cat under dial.

impulse. Such pairs of impulses tend to appear during the period of large, abrupt cortical waves and not in the intervals between the groups. A discharge in which they occur is shown in Fig. 9. The interval between the paired impulses varies between 1 and 10 msec. and is usually in the region of 2.5-3.5 msec.: if there are three impulses instead of two the interval between the first and second is usually shorter than that between the second and third.

The simplest explanation of these multiple outbursts is that they are due to impulses discharged in rapid succession by the unit, the neurones giving a high frequency repetitive discharge instead of a single impulse because the state of excitation is more intense or prolonged than usual. Evidence will be presented later to show that the neurones of the cortex can certainly be made to give short outbursts at very high frequencies, and the present records are probably an example of the same behaviour. But there are other possibilities which cannot be set aside at this stage. One is that the impulses are not all due to a single unit but to several discharging in rapid succession as the wave of activity reaches them.

in single units may have a higher or lower frequency than the cortical waves, but the range of frequency is the same and whenever the cortical waves are regular the correspondence is usually very close. This does not mean that the cortical potentials must be due to the Betz cells, but that in the conditions of our experiments the neurones responsible for the surface waves and those responsible for the pyramidal discharge must become active and inactive in unison. If there were no anaesthetic it is possible that the agreement might be less, though it has not become so under moderately light ether.

We have come to expect a discharge in the pyramidal tract whenever the cortex appears to be in good condition. In some preparations the pyramidal system has been inactive, although potential waves could still be recorded from the surface of the motor area. These have all been preparations in which the cortex has been pale and oedematous as a result of low blood pressure, long exposure, etc. Presumably in such conditions the deeper cell layers may suffer more than the superficial.

Surface potential waves without a pyramidal discharge can also occur during recovery from cerebral anaemia. A temporary failure of cortical activity is produced by occluding the carotid arteries, and when these are released the potential waves in the motor area often return a few seconds before there is any sign of activity in the pyramidal tract. Portions of a record illustrating this are shown in Fig. 13. The cat was under dial and the first effect of carotid occlusion is to increase the frequency of the waves and to make them more continuous (Fig. 13 B). This initial increase in activity nearly always occurs with dial and sometimes with chloralose though not with chloroform and ether. Very soon the cortical potentials and the pyramidal discharge fail simultaneously (Fig. 13 B). Breathing continues since the vertebral arteries supply the medulla, but a collateral circulation is not established in the cortex for several minutes. Fig. 13 C shows the return of activity when the carotids are released 10 sec. after the complete failure of the cortical potentials. It will be seen that there is a period of 2 or 3 sec. in which there are cortical waves but no pyramidal discharge.

If the cortex is deprived of blood for a longer time the whole process of recovery is slower and there is a longer period of dissociation. Thus in the experiment shown in Fig. 14 the carotids were occluded for 35 sec., the cortical potentials reappeared 14 sec. after the carotids were released and the pyramidal discharge did not return until 26 sec. later. The records illustrate another result of cutting down the blood supply, namely the much simpler pattern of the cortical potential waves. The simplification

the pyramidal impulses were slower and less regular. As in the experiments with dial, the correspondence is not due to a direct electrical spread of the cortical potentials, for the medullary record only resembled the cortical when the electrode was in the region of the pyramidal tract.

The appearance of the rapid rhythm in the pyramidal tract is confirmed by records from single units (Figs. 11, 12). These show a continued series of impulses at a frequency in the neighbourhood of 50 per sec., falling to lower values in very deep anaesthesia. The relatively fast rhythm is probably due to a stimulating effect of the ether on the cells

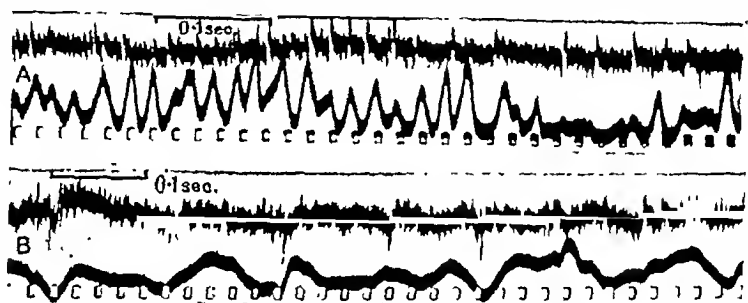


Fig. 12. Records from single unit of the pyramidal tract (above) and from motor cortex (below). A. During C.E. anaesthesia. B. Later under dial. The discharge frequency falls from 52 per sec. with C.E. to 10 per sec. with dial.

of the cortex, for in lighter anaesthesia there is usually an increase in frequency when more ether is given. As with dial, the pyramidal discharge has no very obvious effect on the muscular system, though as a rule the muscles are not completely flaccid until the depth of anaesthesia reaches the stage at which the pyramidal rhythm begins to fail.

As with dial there are sometimes groups of two or three impulses very close together. It is to be noted, also, that the same neurones take part in the 10 per sec. discharge with dial and the 50 per sec. with ether. This may be seen from Fig. 12 which gives two records of the same single unit, the first made during chloroform and ether anaesthesia and showing a rhythm of 52 per sec. in the motor cortex and the pyramidal unit, and the second made later under dial when the discharge rate has fallen to 10 per sec.

(c) *Dissociation of cortical and pyramidal activity*

In the cat we have found no condition in which the discharge in the pyramidal tract, if it occurs at all, is not in reasonable agreement with the potential waves in the motor area. At a given moment the impulses

increasing the depth of anaesthesia and sometimes impossible to do so without abolishing the cortical waves as well.

These results make it clear that the potential waves in the motor area and the discharge in the pyramidal tract are closely related but are not inseparable. It must be remembered also that the agreement is between the general cortical and pyramidal activity: the discharge in a single unit of the tract will not necessarily agree with the potential waves from a limited region of the motor area. In the cat under dial it is true that there is very little difference in the potential record from different parts of the motor area, and the greater the synchronization the better will be the agreement between any part of the motor cortex and any fibre of the tract. In the monkey, where the motor area is much larger, the regional differences are greater and a single unit response from the tract may differ considerably from a record of cortical potentials.

It may be mentioned here that occlusion of the carotids has often been used to decide whether a given discharge in the medulla has come from the cortex or not. The medulla receives its blood supply from the vertebral arteries; thus the sensory pathways in it will be unaffected by carotid occlusion, but a discharge in the pyramidal fibres will be abolished since the blood supply to the cortex will fail. Ultimately, i.e. 10 min. after the carotids are tied, a collateral circulation through the cortex will be established. In one animal (out of 20 or more) it must have been established very rapidly, for carotid occlusion had no effect on the cortical activity or the pyramidal discharge.

(d) *Effects of sensory stimulation*

The activities of the pyramidal system described in the preceding sections have been called spontaneous because they continue in the absence of intentional stimulation. It is true that there must always be some inflow of afferent impulses from the body and we know from the work of Renshaw, Forbes & Drury [1938], and Marshall, Woolsey & Bard [1937] that afferent signals can reach the cortex even in very deep anaesthesia. They may be an essential factor in maintaining the general level of excitation of the brain even when the anaesthesia is deep enough to prevent any modification of the cortical activity by sensory stimuli. In lighter anaesthesia, however, although the animal lies inertly and shows no sign of integrated behaviour, sensory stimuli may cause a definite increase in the cortical waves and an associated increase in the pyramidal discharge which is sometimes enough to cause the movement of a limb.

of the cortical record resembles that occurring with deepening anaesthesia and might very well follow a reduction in the number and complexity of active elements in the cortex.

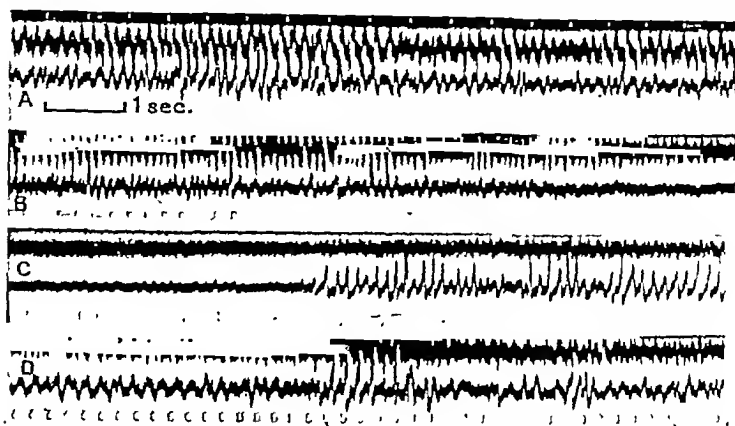


Fig. 13. Dissociation of pyramidal and cortical activity after temporary occlusion of carotids. Cat under dial. Upper tracing in each record from pyramidal decussation, lower from motor cortex. A. Normal. Waves at 8 per sec. B. 10 sec. after carotid occlusion. Discharge frequency increased to 11 per sec. followed by failure. C. Return of activity following release of carotids 10 sec. after failure. Return of cortical waves before pyramidal. Record made 11 sec. after carotid release. D. 30 sec. later. Pyramidal discharge has returned.



Fig. 14. Dissociation of pyramidal and cortical activity. Upper tracing from pyramidal decussation (single unit), lower from motor cortex. Cat under dial. A. Normal. B. Carotids occluded 35 sec. Record 16 sec. after release, showing absence of pyramidal discharge and simplification of cortical waves.

The effect of increasing the depth of anaesthesia is less constant; in some animals a second dose of dial (0.1 c.c./kg. intravenously) has caused a simultaneous failure of both cortical and pyramidal activity, but in two there has been a failure of the pyramidal discharge with retention of the cortical waves. In one of these the pyramidal discharge returned an hour later. The effect of chloroform and ether is equally variable, for it is sometimes possible to abolish the pyramidal activity alone by

With either of these drugs the anaesthesia can be kept at such a level that a slow movement of withdrawal of the limb will usually occur when the foot is pinched. Although the stimulus produces only a localized response and does not modify the state of profound stupor, the pyramidal discharge is probably not far different from the discharges which cause similar movements when the animal is awake. Records of discharges associated with a slow movement of the limb are given in Fig. 17. It will be seen that the impulses in single units form a fairly regular series with a frequency which begins at 10 per sec. or less and may rise as high as 90 per sec. The actual values can be seen more clearly from the curves

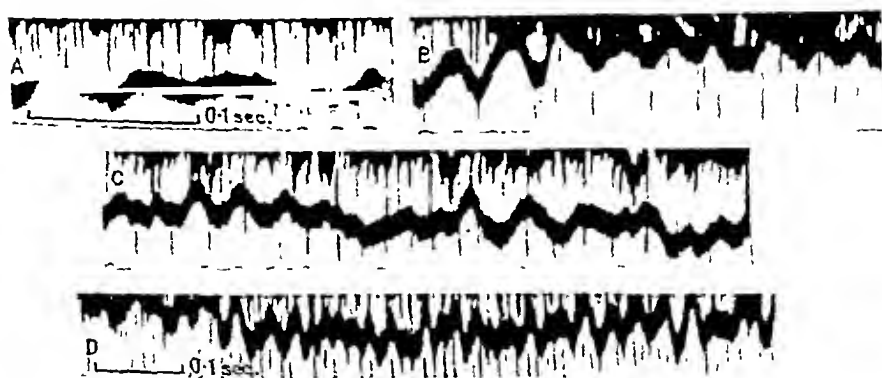


Fig. 17. Single unit discharges from pyramidal decussation (above) and potentials from motor cortex (below) during sensory stimulation leading to movement of a limb. Cat under MgSO_4 anaesthesia. A. No stimulus. Discharge 25 per sec. B. Beginning of stimulation. Discharge rises to 50 per sec. C. Discharge has risen to 80 per sec. D. Slower record, during stimulation. Discharge 90 per sec.

in Fig. 18 which give the frequencies in a single unit during discharges provoked by stimuli of various strengths. There is no tendency towards a characteristic frequency and the variation is quite as great as that in the discharge from a motor nerve cell of the spinal cord.

It might be thought that a rhythmic discharge of impulses at a frequency of 50 per sec. or more would be associated with a steady, instead of an oscillating activity in the motor cortex. But with ether we have already seen that the "spontaneous" cortical potentials agree with the pyramidal discharge up to 60 per sec., and in this activity induced by sensory stimulation there is an equally clear agreement. This is shown in the records in Fig. 17 which give the cortical waves as well as the impulses. As the cortical waves are composite effects they show less regularity than the impulses in one unit, but the rise in frequency

Fig. 15 shows the kind of increase which may be found under fairly deep dial anaesthesia. The record is from a cat with a diffuse lead in the pyramidal tract. In the absence of stimulation the discharge occurs in waves at about 5 per sec. with an occasional faster group, but pinching the foot starts a long series of waves with the characteristic "dial" frequency of 8–10 per sec. It is well known that the electrical activity of the cortex can be increased in similar conditions by sensory stimuli [cf. Bremer, 1938; Adrian, 1939] and the present record merely shows that the Betz cells participate in the increase. In Fig. 16 the anaesthetic

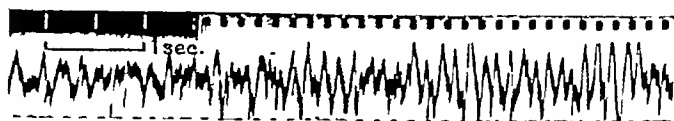


Fig. 15. Increase of pyramidal discharge on sensory stimulation. Cat under dial. Record from decussation. Stimulation (pinching foot) shown by signal above.

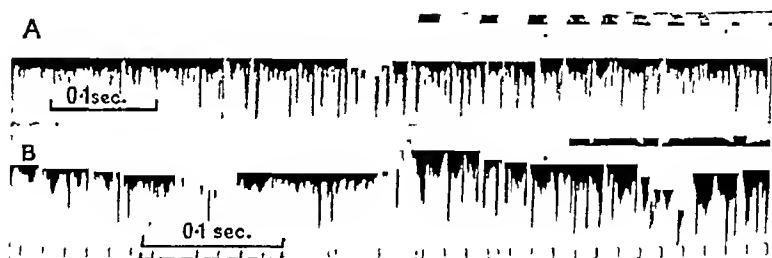


Fig. 16. Single unit pyramidal discharges produced by sensory stimulation. Cats under C.E. In A many of the action potentials are multiple.

was chloroform and ether and the frequencies are higher. In Fig. 16 A the stimulus (pinching the foot lightly) evokes a series of outbursts at 30–40 per sec., most of them of the multiple or repetitive type. In Fig. 16 B the discharge begins more gradually. Neither of these discharges led to any movement, but it is clear from them that the frequency of discharge from the cortical neurones can vary over a fairly wide range and can be increased by afferent impulses.

A nearer approach to the normal can be obtained by using sodium evipan (0.1–0.2 c.c./kg.) and better still magnesium sulphate as anaesthetic.

The anaesthetic properties of the latter were discovered by Meltzer & Auer [1908]: we have used 4 c.c. of a 1 *M* solution subcutaneously to give a deep initial narcosis and have lessened it if necessary by an injection of 2–10 c.c. CaCl₂ *M*/8 intravenously.

of 1/50 sec. or more: in others the number may be higher and the intervals less, and we have twice found the discharge changing gradually into the characteristic high-frequency type to be described later as the result of convulsant drugs. Evidently chloralose has some of the properties of a convulsant besides those of an anaesthetic; for the convulsants also agree with it in making the Betz cells of the anaesthetized brain accessible to sensory stimuli, and Martino [1931] has shown that chloralose facilitates the local action of strychnine on the motor cortex. With chloralose the Betz cells are not all equally affected by sensory stimulation: some may give the abrupt discharge on sensory stimulation, whilst others are quite inaccessible and continue to give a periodic discharge at a low frequency (2-5 per sec.) resembling that with dial.

The mechanical precision with which the jerk reaction can be obtained makes it a simple matter to measure its latency. The moment at which the foot is touched can be signalled on the record by placing a resistance in the earth lead from the animal so as to increase the artefact which arises when the capacity is changed. Records made in this way are given in Fig. 19. That at the top shows the afferent discharge (from touching the forefoot) recorded from the cuneate nucleus after destruction of the motor cortex on both sides. The other records are from pyramidal fibres. The conducting pathway is therefore increased by the distance from the sensory decussation to the motor cortex and from there to the pyramidal decussation, but this alone is not enough to explain the increased latency. As Table I shows, the latencies are remarkably constant from one animal to another, although only about half the time

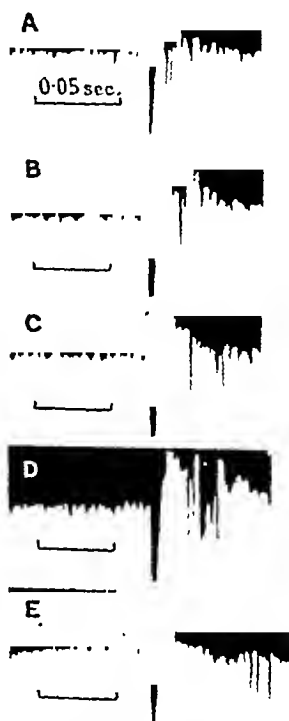


Fig. 19. Jerk reaction in chloralose anaesthesia. Records from the medulla. The moment at which the animal is touched is shown by the artefact in each record. A. Sensory impulses recorded from the cuneate nucleus after bilateral destruction of motor cortex. Latency 8.6 m.sec. = conduction velocity 35 m./sec. B. Another animal. Pyramidal impulses from left motor cortex. Touch to right forefoot. Latency 18 m.sec. C. Ditto. Touch to left forefoot. Latency 24 m.sec. D. Impulses from right motor cortex, touch to right forefoot. Latency 22 m.sec. E. Impulses from left cortex, touch to left knee. Latency 56 m.sec.

on stimulation is well marked. A detailed comparison of the cortical and pyramidal responses promises to give interesting results in several directions; for the present, however, we are concerned only with the fact that there is a parallel variation in frequency with sensory stimulation.

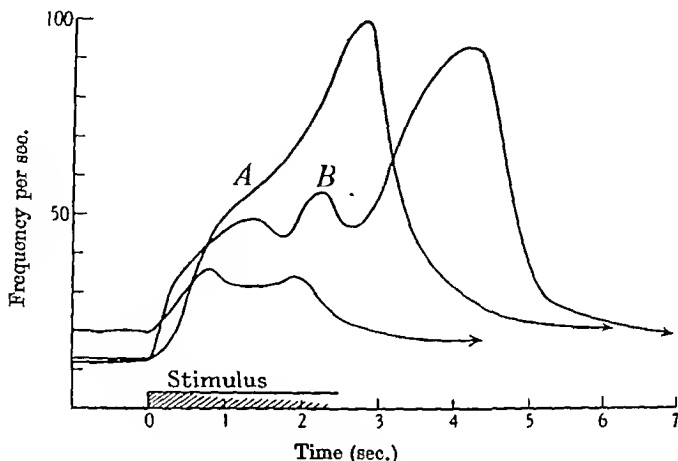


Fig. 18. Curves showing frequency of impulses in single units during discharges induced by sensory stimulation. Those marked *A* and *B* were accompanied by limb movement. Cat under MgSO_4 anaesthesia.

Sensory stimulation in chloralose anaesthesia. Sensory stimulation gives a much more constant and striking effect when the animal is under chloralose (35 mg./kg.). The familiar effect of this drug is to produce a combination of deep anaesthesia with increased responsiveness to an abrupt stimulus such as a light tap on one of the feet or even on the table near the animal. This evokes a sudden movement, sometimes restricted to the limb which was touched but often a convulsive jerk involving all four limbs and the trunk as well. The reaction fatigues if repeated at short intervals and varies from time to time without obvious cause: in addition there is often a varying degree of tone in the muscles.

The convulsive response to a touch is a cortical reaction. We find that destruction of both motor areas abolishes the response, leaving only some increase in the limb reflexes, and that temporary occlusion of the carotids produces a temporary failure; also, whenever the jerking movement occurs there is an abrupt potential wave in the motor area and a corresponding discharge of impulses in the pyramidal tract. The frequency of the impulses in this discharge can vary widely. In some preparations there are not more than two or three impulses in each unit at intervals

experimental purposes it has the advantage that it can be obtained repeatedly and has the same characters from one animal to another. Records showing both the primary (jerk) and the more deliberate secondary discharge are given in Figs. 20 and 21. In the former it will be seen that the frequency varies widely with the intensity of the stimulus, in the latter with the degree of fatigue. The variation is also shown by the frequency curves in Fig. 22.

With the movements obtained in eripan and $MgSO_4$ anaesthesia it was found that the motor cortex gave a potential oscillation with a

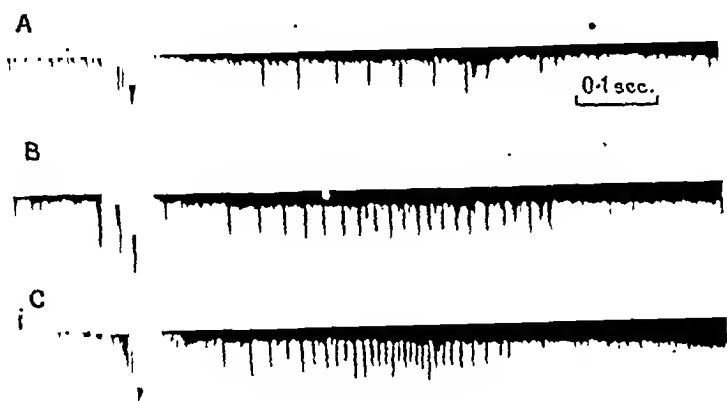


Fig. 20. Records from the pyramidal decussation in chloralose anaesthesia showing "primary" and "secondary" discharges in response to tactile stimuli of different intensities. A. Weak stimulus. Secondary response begins after 0.2 sec. latency. Frequency rises to 27 per sec. B. Stronger stimulus. Latency 0.16 sec. Frequency rises to 60 per sec. C. Very strong stimulus. Latency 0.15 sec. Frequency rises to 130 per sec.

frequency comparable to that of the pyramidal discharge. In the secondary chloralose response we have not been able to detect any clear cortical effect. The primary jerk reaction is accompanied by a large diphasic wave in the cortex but as the secondary discharge takes place in relatively few units the cortical effect is bound to be much smaller. The two records in Fig. 23 give corresponding cortical and pyramidal responses, but they are chiefly noteworthy in showing the complex initial wave which probably indicates both the arrival of the sensory volley (+ component) and the activity of the superficial layers of the cortex (- component).

TABLE I

Exp.	Site of stimulus	Latency (average) to m.sec.	Length of pathway cm.	Conduction velocity m./sec.	Synaptic delay m.sec.
a	Forefoot	Cuneate nucleus 7.8	30	38	Nil
b	Contralateral forefoot	Pyramidal decussation 22.8	42	40 (assumed)	12.3
c	Contralateral forefoot	— 18	—	—	7.5
d	—	— 17.3	—	—	6.8
a	—	— 20	—	—	9.5
d	Ipsilateral forefoot	— 20.5	—	—	10
c	Contralateral hindfoot	— 28	72	—	10
d	Ipsilateral knee	— 50.4	57	—	36.2

seems to be taken up in the conduction of impulses to and from the brain. This may be seen from the last column in Table I which gives the delay assuming a velocity of conduction of 40 m./sec. (cf. Fig. 19 A). On this assumption the total synaptic delays are of the order of 10 m.sec. when the forefoot is stimulated. When the hindfoot is stimulated they are usually the same but occasionally much larger, as in Fig. 19 E. The delay may increase if the reaction is evoked repeatedly at very short intervals, but it may be necessary to stimulate as often as six times a second to cause an appreciable change.

In deep chloralose anaesthesia no movement occurs on stimulation but the abrupt pyramidal discharge is still evoked with mechanical precision and a fairly constant latency. It is evidently a cortical activity of abnormal type. In lighter anaesthesia, however, the initial outburst is often followed by a longer series of impulses with a gradual rise and fall of frequency. This secondary discharge with chloralose, like the discharges accompanying movement with evipan, etc., is much less able to tolerate adverse conditions. Occlusion of the carotids abolishes it immediately, although the primary (jerk) discharge continues for 10 sec. or more before it fails completely. Again, the secondary discharge is much more easily fatigued when the stimulus is repeated at short intervals. It has not been possible to decide whether it is accompanied by any movement, for the powerful jerk would overshadow a subsequent contraction. But it is so much like the pyramidal discharges which produce slower movement that it deserves to be considered as an approach to the normal activity of the pyramidal system, and for

or more and careful searching may give a record of the single impulses. Records made in this way may be complicated to some extent by the large cortical potentials, but have shown all the features of the pyramidal

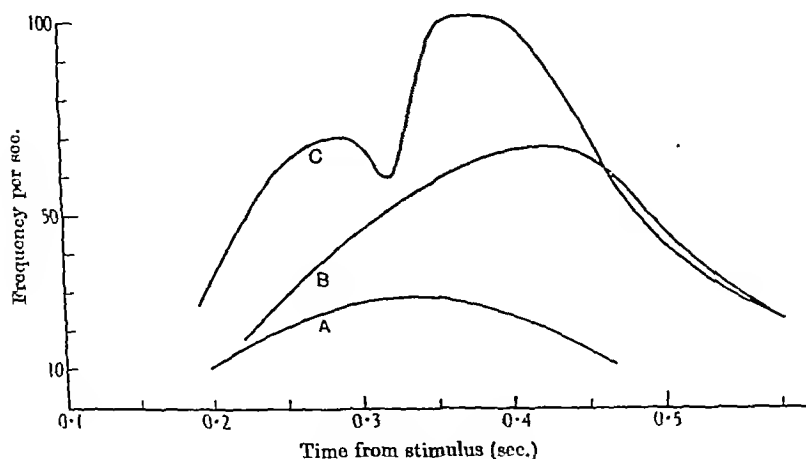


Fig. 22. Frequency of impulses in single unit of pyramidal tract during the secondary response under chloralose. Tactile stimuli of different intensities

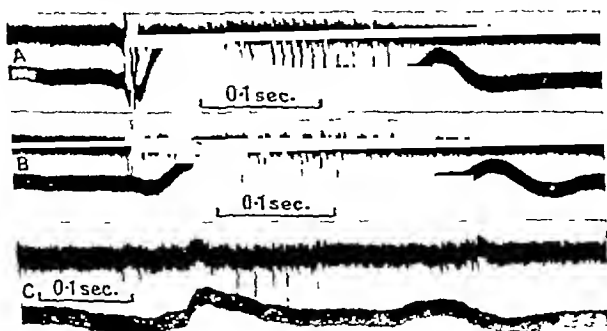


Fig. 23. Pyramidal discharge from the decussation (upper tracing) and potentials from motor cortex (lower tracing) in chloralose anaesthesia. A. In response to touch (strong). B. Ditto, weaker stimulus. C. Another cat, spontaneous discharge with no movement. In A and B the initial cortical waves are diphasic. Downward movement indicates positive potential.

discharge as it appears in the medulla, e.g. the typical frequencies with dial and ether, the multiple outbursts and the increase in some cases with sensory stimulation.

(e) Records from the white matter of the cortex

The fibres which go to form the pyramidal tract should be accessible to an electrode in the motor cortex as well as to one in the medulla. If

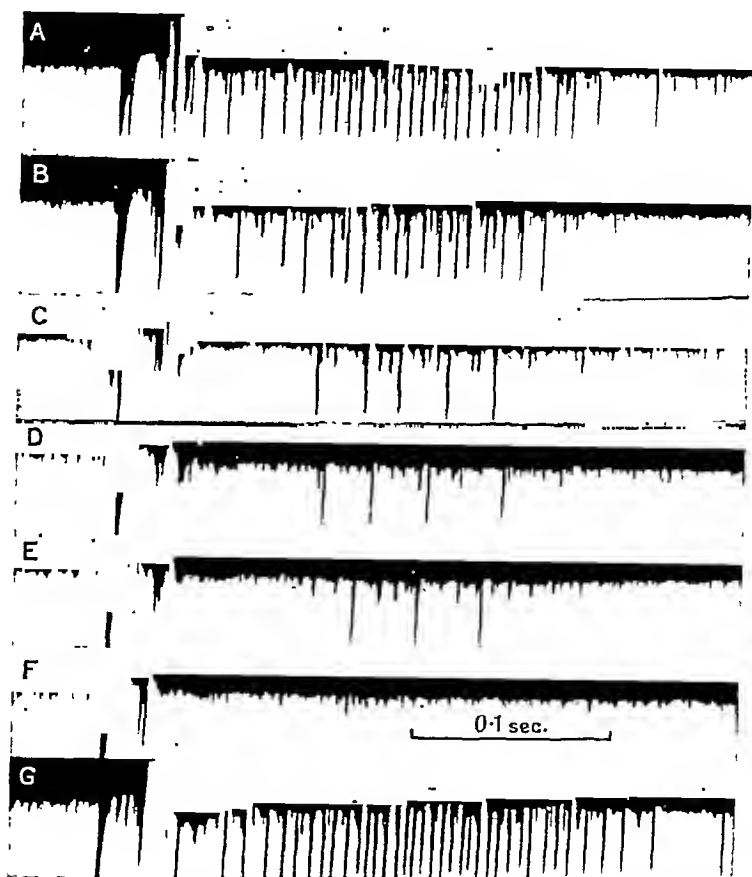


Fig. 21. Records from the pyramidal decussation showing the effect of fatigue on the chloralose response. Discharges evoked by tapping the foot repeatedly. Records A-F made at $\frac{1}{4}$ sec. intervals, stimulus marked by artefact. With repetition the latency of the primary response does not alter, but that of the secondary response is increased. The latter declines in frequency and duration and ultimately disappears (F). After a pause of 10 sec. without stimulation the secondary response appears again (G), the frequency rising to 190 per sec.

a fine enamelled wire is thrust into the sigmoid gyrus of a cat under dial the characteristic discharge can usually be detected at a depth of 3 mm.

(f) High-frequency outbursts due to strychnine

In some of the earlier experiments strychnine solution was used as a convenient means of stimulating the motor cortex. The method was introduced by Baglioni & Magnini [1909] and has been employed recently by Dusser de Barenne [1916, 1924]. It consists in applying a small pad of filter paper or cotton-wool soaked in a 1 or 0.1 % solution to the surface of the brain. In a cat under dial the electrical activity of the affected region changes in a few minutes to groups of a few very large potential waves separated by quiet intervals, and if the motor area has been treated corresponding potential waves are to be found in the pyramidal

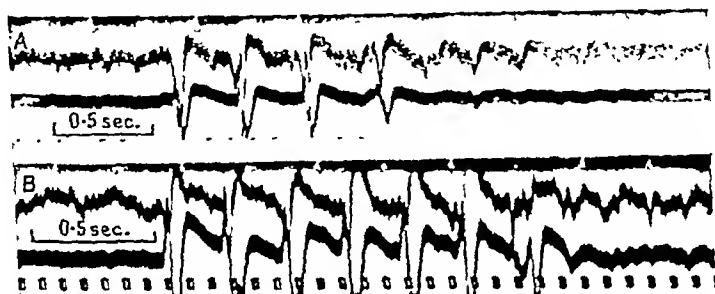


Fig. 24. Discharges due to local application of strychnine to the motor area. Cats under dial. Upper tracing gives pyramidal discharge recorded from the medulla, lower gives cortical potentials. Large waves occur in groups at 4 per sec.

tract (Fig. 24). In our early experiments the finding of these typical strychnine waves in the record from the medulla confirmed the view that we were dealing with the pyramidal system.

In later experiments, when the medullary electrode was arranged for recording from a very few units instead of the whole tract, it was found that each major wave of the strychnine discharge is made up of a large number in impulses following at very short intervals. The records show a marked resemblance to those of strychnine discharges in peripheral nerve made by Rosenberg & Kitayama [1930]. In these Rosenberg found groups of regular potential waves at very high frequencies, and concluded that the units must be discharging in rotation or else at much higher rates than usual. In the pyramidal tract the electrode can usually be arranged to give single unit records, and it can then be seen that the impulses occur in definite sequences at rates of 500 per sec. or more. The high frequency is confirmed by the noise of the discharge in the loud-speaker which rises to a squeak. It is difficult to obtain good high-speed

Less operative interference is needed to obtain records from the white matter of the cortex than from the medulla, but all such records have one great disadvantage, namely that we cannot be sure that axon potentials in the motor area come from pyramidal fibres unless they have been produced by stimulation of the area, for the white matter of the sigmoid gyrus contains afferent fibres as well as efferent. It is probable that the discharges which we have recorded in the white matter have all come from neurones in the cortex, since they have not differed appreciably from the pyramidal discharges, but they may have started from other regions than the motor area. There are in fact no clear-cut differences in the electrical activity of different regions of the cat's cortex under dial or ether, and we have recorded the 10 per sec. or the 50 per sec. impulse discharge in the white matter of several regions some way from the sigmoid gyrus.

Another disadvantage of recording from the white matter of the cortex is that the electrode may have injured the region from which the impulses arise. A relatively thick wire (e.g. No. 40 s.w.g.) as it penetrates the grey matter is almost certain to cause injury discharges of the kind described by Adrian & Matthews [1934]. These subside after a minute or less, but the effects of the injury may remain. With thinner wires there is usually no disturbance when the wire is inserted, but a local injury might be the cause of one feature in which these records differ slightly from those made from the pyramidal tract. This feature is the more frequent appearance of multiple outbursts instead of single impulses. The difference is one of degree only, but it raises again the question as to the nature of the multiple outbursts. The following sections give more definite information on this point, for they describe the much longer outbursts caused by convulsive drugs and by electrical stimuli.

PART II. HIGH-FREQUENCY OUTBURSTS IN THE PYRAMIDAL DISCHARGE

This type of discharge, in its fully developed form, is never found except as the result of abnormal or pathological conditions. Yet there are two reasons which make it worth considering in some detail. One is that it may throw some light on the cell mechanisms of the cortex, the other that it is almost certainly the type of discharge responsible for epileptiform effects. It is at least responsible for the convulsions of cortical origin which can be produced experimentally by drugs or electrical stimuli. Thus the high-frequency outburst has a strong claim to be considered an essential part of the phenomena of certain forms of epilepsy.

are some in which the frequency declines steadily to values of 50 per sec. or less (cf. Fig. 32).

As with the multiple outbursts in the normal spontaneous discharge (p. 162), the most straightforward explanation of this grouping of impulses is that they represent a repetitive discharge from a single nerve cell or from a group of nerve cells acting as a unit. The discharge might conceivably be due to re-excitation at short intervals through a neurone circuit of the type postulated by Lorente de Nó [1938], but in view of the high frequencies it is simpler to assume that the drug has produced an abnormal prolongation of each period of activity in the Betz cells, so that they discharge a series of impulses instead of a single one (cf. Fig. 27). An increased intensity of activity without change of time relations might account for the groups of two or three impulses very close together, but it could scarcely account for outbursts maintained at a

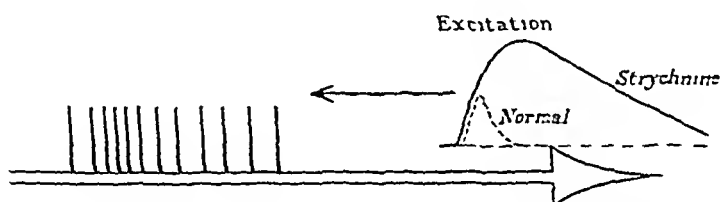


Fig. 27. Suggested production of high-frequency outburst by prolonged activity in a single unit.

high frequency for as long as 10 sec. (as in Fig. 30). To explain these we must assume a slowing of some process which normally brings each period of activity to an end after the discharge of a single impulse. The process need not be specified: there may be a slower repolarization of active dendrite surfaces, the axon may take longer to become adapted to the excitation of the cell body, or a chemical excitant may be destroyed more slowly [cf. Nachmansohn, 1939]. If the discharge becomes repetitive owing to changes of this kind, the actual range of frequency would be governed by the absolute and relative refractory periods of that part of the neurone in which the repetitive response is set up. The intervals between successive impulses are in fact of the same order as in the repetitive discharge from injured mammalian nerve fibres [Adrian, 1930], and might well be determined by the recovery time of the axon.

Before this "single unit" explanation can be accepted there are the two other possibilities to consider. One is that the high-frequency outbursts are due to a number of units discharging in rotation; the other

photographs of the impulses, since the exact moment at which each discharge will occur is uncertain. Thus we have not tried to use recording speeds greater than 40 cm./sec., although we have made many visual observations with the rotating mirror giving speeds equivalent to several metres per sec.

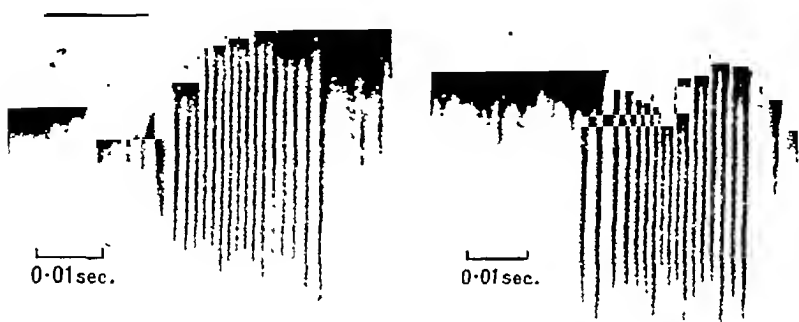


Fig. 25. Typical "high-frequency outbursts" due to local application of strychnine to the motor area. Records from decussation showing two consecutive outbursts. Each group of action potentials would correspond to one of the waves in Fig. 24. Maximum frequency 890 per sec.

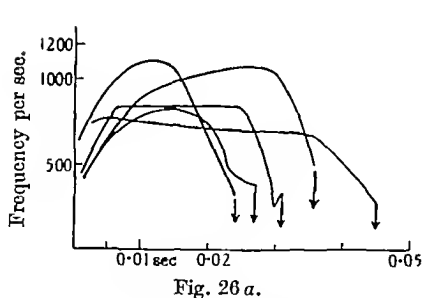


Fig. 26 a.

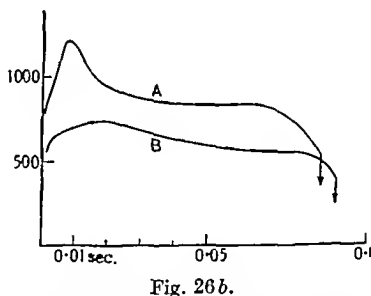


Fig. 26 b.

Fig. 26. Frequency of impulses in single units in various high-frequency outbursts due to strychnine, and in one (B) occurring in a preparation under chloralose. The discharge marked A was recorded from the white matter of the motor cortex. The others are from the pyramidal decussation.

Fig. 25 gives two enlargements of high-frequency outbursts in which most of the impulses belong to a single series, and the curves in Fig. 26 show the frequency of the impulses at different times after the start of the discharge in various experiments. The curves take different forms, rising and falling sharply or slowly. The shortest interval between successive impulses has not been less than 0.6 m.sec.; in most of the outbursts the longest interval is of the order of 4 m.sec., though there

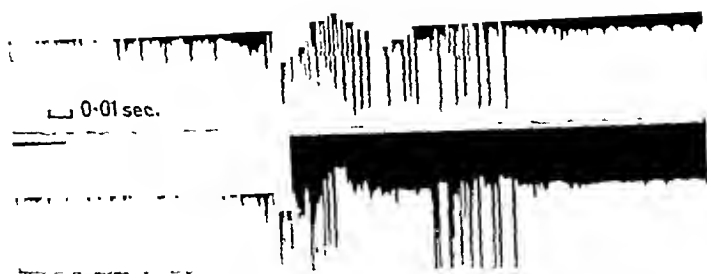


Fig. 29. High-frequency outbursts due to strychnine, showing at least two units discharging in association. Both records from the same preparation, illustrating tendency to repeat the same pattern of discharge.

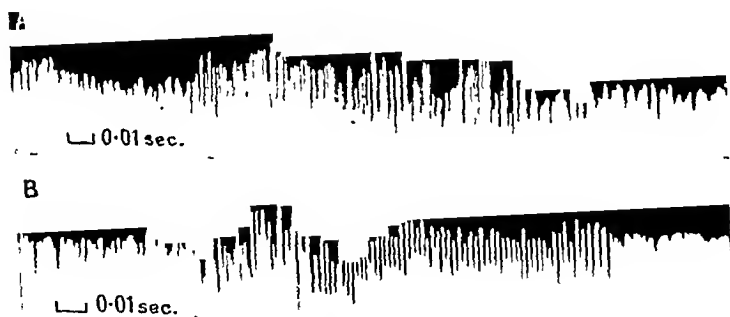


Fig. 30. Outbursts in pyramidal decussation. A. Due to local application of picrotoxin (crystal) on the motor cortex of a cat under dial. B. Occurring spontaneously in a cat under chloralose. Both associated with convulsive movement.

is that they are due to injury caused by the wire electrode. The first possibility is indicated in Fig. 28. It does not raise a very serious issue, for it demands a group of neurones acting in such close association that the group might almost be thought of as a single unit. However, it is difficult to believe that there would not be some progressive change in the size of the successive action potentials if each one came from a different fibre. It is not unusual to find a regular series in which the spikes are of two distinct sizes. Figs. 29 and 31 are good examples, but in all of them the large spikes alternate with the small as they would do if the "unit" was made up of two groups of neurones discharging in phase, but one at half or a third the frequency of the other. Records with two or more impulse sequences to each outburst are also common enough, and in any

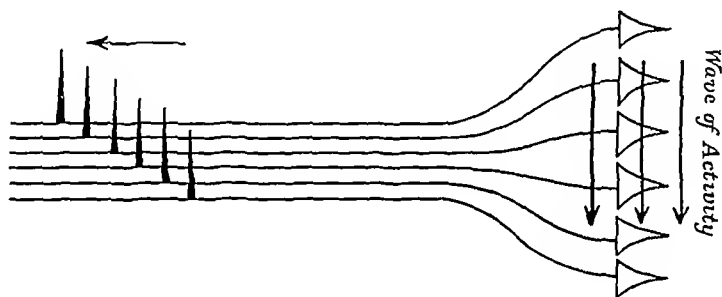


Fig. 28. "Multi-unit" hypothesis: production of high-frequency outburst by units discharging in rotation.

record there are random movements of the base-line which make some variation inevitable. But we have never observed a regular sequence of potentials with a progressive change in size and this should certainly occur if each of them is due to a different fibre.

Another argument for the single unit explanation is the way in which the frequency rises and falls in each outburst, as in the curves in Fig. 26. This can be simply explained as the result of a parallel change in the intensity of excitation in the neurone, but on the multiple unit hypothesis there is no explanation. The rise and fall of frequency must be due to changes in the rate of conduction of the excitation from one unit to the next in the series, but there is no obvious reason why the intervals should vary as they do or why they should be confined to a particular range.

The range is in fact rather low to be explained on the simple hypothesis of a wave of activity spreading over the cortex and causing a group of neurones to discharge in succession. Certainly the potential waves in the cortex are not stationary, but they seem to spread

too fast to give the right interval between one impulse and the next. In the rabbit with electrical stimulation the waves move at 50–20 cm./sec.: in the cat under dial the velocity of the spontaneous waves cannot be estimated so accurately as they do not move so far, but it seems to be of the same order. In sections of the cat's motor area we find the average distance between the Betz cells to be 90μ . Thus a wave of activity travelling at 20 cm./sec. along a line of cells should make them discharge at intervals of $90/200 \times 1000 \text{ sec.} = 0.45 \text{ m.sec.}$, and to produce frequencies as low as 200 per sec. the wave would have to move at 2 cm./sec. instead of 20 cm. The Betz cells are crowded in some places and infrequent in others: a wave of activity spreading through a crowded area might give a succession of impulses with an increasing and then diminishing frequency, but again the frequencies would be too high unless the wave travels very slowly, for in the crowded areas the Betz cells may be only 10μ apart. It might be added that the Betz cells are never spaced as regularly as are the impulses in the typical high-frequency outburst, though it is true that irregular spacing would not involve an irregular succession of impulses if the rate of spread of the wave is determined mainly by synaptic delay between one neurone and the next. Obviously by suitable modification the multiple unit hypothesis could be made to agree with many of the facts, but at least it gains no support from existing numerical data.

The other possibility which needs to be examined is that the high-frequency outbursts are the result of injury by the electrode which is employed to record them. This would seem plausible in the case of those outbursts which are recorded from the white matter of the cortex, for the passage of a wire electrode through the grey matter does undoubtedly set up injury discharges and the strychnine might exaggerate the effect; with the wire in the medulla, however, it is difficult to see how injury to the fibres there could interact with the strychnine excitation of the cortical cells. But there is at least a *prima facie* case for injury as a factor in the discharge, since the spacing of the impulses often resembles that in the injury discharge from a nerve fibre. Moreover, it is conceivable that the very large potential waves in the cortex might cause electrotonic changes which would spread some distance down the pyramidal fibres.

To decide the point an experiment was made in which the strychnine discharges were recorded without using an electrode which could injure the pyramidal fibres. The ventral surface of the medulla was exposed by cutting through the basal part of the occipital bone, so that the two uncrossed pyramidal tracts could be seen lying superficially on either side of the midline. A cotton thread electrode was brought into contact with one tract and strychnine solution was applied to the motor cortex on the same side. In a few minutes the typical strychnine discharge could be recorded from the pyramidal fibres and the electrode was then moved to the extreme outer border of the tract so as to avoid the main bulk of the fibres. In this position, although the response came from many units, it was possible to detect the characteristic high-pitched squeak in the loud-speaker. The fibres were uninjured but the outbursts

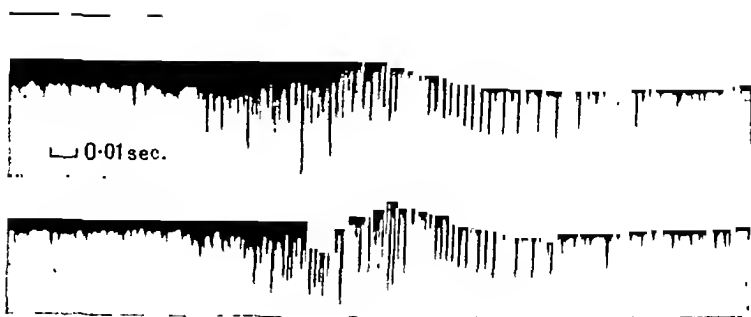


Fig. 32. Complex (multi-unit) outbursts after local application of strychnine, showing final series with frequency falling to 50 per sec. Both outbursts are due to sensory stimulation (touching foot) and show the tendency to repeat the same pattern of discharge (cf. Fig. 29).

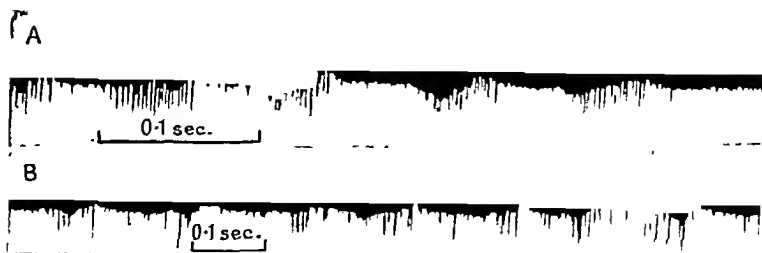


Fig. 33. Final stage of local strychnine effect in a cat under dial. A. High-frequency outbursts occurring in groups at 10 per sec. B. A few minutes later the usual 10 per sec. discharge has reappeared.

cortex had been exposed and may perhaps have been damaged, for as a rule chloralose does not produce the typical high-frequency outburst except as the result of sensory stimulation.

Fig. 31 is an outburst due to a small crystal of eoryamyrtin on the cortex and is particularly interesting as evidence that several fibres may combine to give the single unit type of discharge. The potentials which make up the outburst are evidently produced by at least two neurones or groups of neurones acting in very close association. In one of these the impulses have a maximum frequency of 1000 per sec.; the other discharges in phase with it but cannot reach such high frequencies. Thus for the greater part of the outburst the frequency in the second is half that in the first, so that the potentials are alternately small and large. At the beginning and end the frequency in the first is low enough for the second to agree with it and all the potentials are large. In this case, therefore, at the lower frequencies the unit consists both of the pace-maker and of the slower neurones which are driven by it. A similar alternation of small and large potentials can be seen in many of the high-frequency outbursts (cf. Fig. 29), though the large potentials are not spaced so regularly.

(h) *Development of high-frequency outbursts*

Further light is thrown on the nature of these outbursts by studying their development when a convulsant drug is applied to the cortex. In an animal under dial the outbursts often appear suddenly without any increase in the frequency of the spontaneous discharge. With strychnine, however, there may be an increase in the number of units in action and in the electrical excitability of the motor area, as shown by the reduction in the strength of shock required to set up impulses in the pyramidal tract. The characteristic, abrupt discharge is first noticed as a result of sensory stimulation and later as a spontaneous effect (i.e. without intentional stimulation). The outbursts then occur in groups of three or four every few seconds and continue in this way for long periods. As time goes on there is often a progressive increase in the maximum frequency in the outbursts, e.g. from 500 to 1000 per sec., and it is our impression that in the earliest stages the discharge may consist of only two or three impulses in each unit at a relatively low frequency. Similar discharges occur in some units throughout the period of high-frequency outbursts in others, but it is difficult to secure good records of the former, for with many units in action it is much easier to follow the long high-frequency sequences.

seemed to differ in no way from those recorded in other experiments by the wire electrode. For the high frequencies in each unit we were compelled to rely on our interpretation of the loud-speaker record, and as this was the composite noise produced by a number of units the evidence is scarcely conclusive. But the duration of each discharge period was certainly as long as in experiments where the wire was used and the noise gave no suggestion of a lower frequency. We conclude, therefore, that injury by the electrode is not the factor responsible for the arrangement of impulses in the strychnine outburst.

(g) *Other convulsant drugs*

Experiments with other convulsants have shown the same type of high-frequency outburst. We have used cardiazol and thujone injected

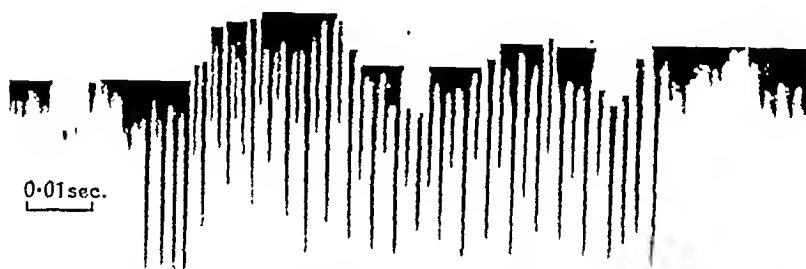


Fig. 31. Outburst due to local application of coryamyrtn (crystal) to the motor cortex, showing small and large potentials alternating when the frequency exceeds 800 per sec.

intravenously and picrotoxin and coryamyrtn applied locally to the motor cortex. With injections the activity is usually so widespread that it is difficult to make good records, but the loud-speaker has given the characteristic noise of the high-frequency discharge at each period of discharge. Application of the drug to the cortex has been more successful, for crystals of picrotoxin or coryamyrtn can be used like strychnine to produce a localized excitation of some part of the motor area and the high-frequency outbursts will then continue for half an hour or more. Discharges produced in this way are shown in Figs. 30, 31. Though there are potentials of several sizes, it is possible to make out various regular sequences with the same frequency range as in the strychnine outbursts. Fig. 30 B is an example of an outburst due to chloralose. The motor

every few seconds, they can be evoked at much shorter intervals by tactile stimuli to the forefoot (on the side opposite the affected motor cortex). How rapidly the outbursts can be produced by stimulation depends on the depth of anaesthesia, the limit varying from 6 to 2 per sec. When they are made to occur at or near the maximum rate the impulse frequency and the duration of each outburst may be diminished: apart from this the outbursts are alike whether they occur spontaneously at long intervals or follow repeated tactile stimulation.

In view of all these results we may regard the high-frequency outburst as the characteristic response of a poisoned neurone, equivalent to the single impulse which would normally result from a wave of excitation. The equivalence sometimes appears very clearly when the effect of the drug is wearing off and the cortical response begins to revert to the usual groups of waves at 10 per sec., for in some of the neurones the discharge will consist of single impulses and in some of high-frequency outburst grouped in the same way. An example of the latter type of activity is shown in Fig. 33 A. In the record below (Fig. 33 B), made a few minutes later, the outbursts had given place to the usual 10 per sec. discharge.

(j) *Motor effects*

Except in the deepest anaesthesia, when high-frequency outbursts have begun to occur spontaneously some movement is usually visible on the corresponding side of the body. When the drug is applied locally to the motor cortex the amount of movement is at first very small, since the effects are confined to a small area. With a crystal of picrotoxin, for instance, there may be at first no more than a slight twitch in one of the shoulder muscles corresponding to each discharge, but later when the excitation has spread the movement will be a powerful jerk of the whole forelimb. If precautions have been taken to avoid absorption of the drug by the blood stream, the movements cease at once when the motor cortex is destroyed.

The association of convulsive movement with the high-frequency pyramidal outbursts is so constant that there can be no doubt of the effectiveness of this type of discharge in overcoming the synaptic resistances of the spinal cord. This is confirmed by the experiments to be described in the following section which deals with electrical stimulation of the cortex.

In the later stages a record will often show several units starting to discharge together, and giving outbursts of the usual high-frequency type followed closely by one in which the frequency starts at 3-400 per sec. and falls as low as 50 per sec. (cf. Fig. 32). These records and those in Fig. 29 illustrate a characteristic feature of the multi-unit discharges, namely the tendency for the different impulse sequences to appear in the same order each time the discharge occurs. This would be the natural result of a wave of excitation spreading over the cortex in the same way on each occasion. We have not tried to follow the potential waves in the cortex corresponding to these particular discharges, but records made previously with multiple leads have shown complex wave patterns repeated many times with little variation.

(i) *Factors affecting the outbursts: sensory stimuli*

The high-frequency outbursts, when fully developed, seem to represent an explosive all-or-nothing response of the neurones affected by the drug. They can be made to appear at longer or shorter intervals, but the duration of each outburst and the frequency of the impulses in it is more difficult to change. The frequency can, of course, be altered by lowering the temperature of the cortex. Thus in one experiment the maximum frequency of a particular series was reduced from 650 to 320 per sec. by irrigating the surface of the motor area with cold saline. On the other hand, a reduction of the blood supply (by clamping the carotids) increases the interval between the outbursts and may stop them altogether without any preliminary stage in which the impulse frequency is reduced.

Individual outbursts can be evoked by sensory stimuli in deep dial anaesthesia, although before the strychnine was applied sensory stimulation had no effect on the pyramidal discharge. It was shown by Amantea [1915, 1921] that after application of strychnine to the motor cortex sensory stimulation (contralateral) produces localized clonic movements and sometimes generalized epilepsy. It was shown later that after intravenous injection [Fischer & Löwenbach, 1934] or local application [Bremer, 1936; Gozzano, 1936] of strychnine the cortex will often respond to sensory stimulation with one or more of the characteristic large potential waves. The same result has been found in the present experiments when a convulsant drug is applied locally to the motor cortex, for then each stimulation of the contralateral skin gives a high-frequency outburst in the pyramidal tract. In the early stages of the drug action and sometimes in very deep anaesthesia this may be the only way in which the outbursts can be produced: in the later stages, when they occur spontaneously

shock must be perceptible to the tongue but need not be at all painful. The effect is illustrated in Fig. 34, and it can be seen from this that the group of discharges evoked by a stimulus does not differ appreciably from the group occurring spontaneously. The only difference is that if the stimuli are repeated at short intervals (a sec. or less) some may have no effect and the groups which are set up are shorter than usual. The same result had been found previously for the cortical waves [Adrian 1939].

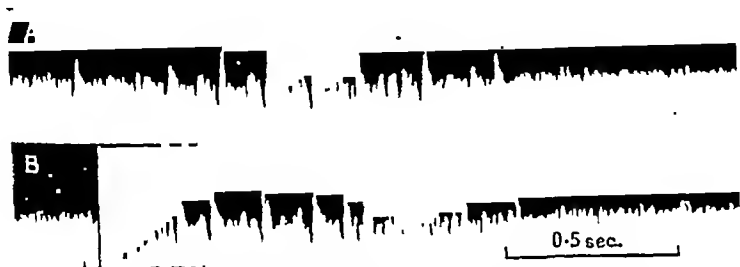


Fig. 34. Pyramidal discharge started by single stimulus to the motor cortex. Cat under dial. Records from pyramidal decussation. A. Group of discharge waves occurring spontaneously (cf. Fig. 3) at a frequency of 7 per sec. B. Group started by single condenser shock (shown by artefact), same frequency as A.

Records made from a very few units show that the impulses are spaced in the same way at intervals of $1/10$ sec. whether the discharge is spontaneous or is evoked by a stimulus. In neither case is there any movement or change of tone to correspond to the pyramidal discharge. With chloralose, however, and in animals under dial when a convulsant drug has been applied to the cortex, a single shock has the same effect as a sensory volley, giving a high-frequency pyramidal outburst and an abrupt jerk of the muscles.

(1) *Production of movement by repeated shocks*

As a rule, to cause movement the cortex must be stimulated many times at short intervals in order to build up the requisite degree of facilitation. This is a process which takes place in the cortex itself [Graham Brown, 1916] and is associated with a progressive increase in the size of the potential wave following each shock [Adrian, 1936]. How many shocks are needed to cause movement will depend on their strength and frequency and on the depths of the anaesthesia [Cooper & Denny-Brown, 1927].

PART III. ELECTRICAL STIMULATION OF THE MOTOR CORTX

The results described in Part I make it necessary to find some new explanation of the fact that electrical stimulation of the motor area can produce movement in the anaesthetized animal. Previously this fact could be accepted without comment, for in the absence of stimulation there was no reason to suppose that the Betz cells were not at rest. But we now find that there is a periodic discharge of impulses down the pyramidal tract although the animal is anaesthetized and the cortex is not stimulated. As these impulses do not produce movement, the discharge set up by electrical stimulation must differ in some way from that occurring spontaneously.

There are two obvious differences which might be invoked to explain the results. In the first place electrical stimulation usually implies a frequency of at least 20 per sec., twice that of the spontaneous activity under dial. The second difference is that with electrical stimulation a large number of the Betz cells will discharge simultaneously, so that impulses from many units will converge on the motor nerve cells of the cord. With the spontaneous discharge we do not know how many units are likely to be in action together, but the number may well be small.

There is, however, a third reason for the production of movements: we find that with repeated shocks the impulses may be discharged in a succession of brief high-frequency outbursts, one to each shock, and it has already been shown that this type of discharge is effective when the low frequency is not. Probably electrical stimulation succeeds for all three reasons, but before we consider how it can cause movements there is another, less obvious, result of stimulating the cortex which must be mentioned briefly.

(k) *Activity following a single shock*

It was shown by one of us that a single electric shock to the cortex of an animal under dial will start a group of potential waves at 7-10 per sec., resembling the groups which occur spontaneously. In certain conditions sensory stimulation may have the same effect [Bremer, 1937], for presumably the waves begin whenever the level of excitation in the cortex reaches a certain height and it may be raised either by afferent impulses or by a direct stimulus. We have recorded the pyramidal tract discharge when the motor cortex is stimulated by a single shock (from a 0.01 mF. condenser charged to 20-50 V.) and have found the expected result, namely that the shock starts a series of impulses in the pyramidal fibres as well as a series of potential waves in the cortex. To do so the

that already found with convulsant drugs. In more excitable preparations weak stimulation will produce single impulses from the beginning, and if the frequency of stimulation is high (e.g. 60 per sec.) movement will begin; if it is low (under 30 per sec.) movement will not begin until the single impulses have been replaced by groups of two or three. The change may be brought about by waiting for facilitation to occur or by increasing the strength of the shocks. In Fig. 36, for instance, the first half shows the effect of stimuli just strong enough to set up impulses. Each shock artefact is followed by a single impulse after a latency of 4.5 m.sec. The second half shows the effect of increasing the strength of the stimuli to twice the threshold value. Each shock now gives two and occasionally three impulses about 1.5 m.sec. apart, the first occurring after a latency of only 2.3 m.sec. With this discharge a movement of the forelimb began almost at once, whereas there was no movement with the

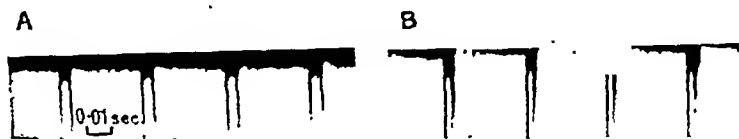


Fig. 36. Production of multiple impulses by stronger stimuli. Cat under evipan. A. Stimuli of threshold strength. Each produces an artefact followed by a single impulse (latency 4.5 m.sec.). No movement. B. Stimuli doubled in strength. Each gives two impulses (latency 2.3 m.sec.). Extension of forelimb.

single impulses. In another preparation stimulation at 60 per sec. gave movement with stimuli not strong enough to set up more than one impulse, but when the frequency was reduced to 30 per sec. the stimuli had to be made 1.8 times as strong. At this strength each shock set up three and sometimes four impulses in the unit and movement occurred.

The obvious conclusion from these results is that stimulation of the motor cortex of a relatively low frequency may cause movement by making the Betz cells discharge short outbursts of impulses at a much higher frequency. These are more effective than the single impulses of the spontaneous discharge or the single impulses set up by the stimuli before facilitation has occurred. It is true that facilitation, besides causing multiple discharges in each unit, will lead to an increase in the number of units in action. No doubt this contributes as much towards the overcoming of synaptic resistances in the spinal cord, for there is probably a considerable degree of convergence of the pyramidal fibres on to the motor nerve cells. But it is interesting to find that very weak stimulation,

When the pyramidal discharge is recorded from the medulla it is often found that the stimulation must be continued for several seconds before impulses begin to appear regularly, and that there is then a rapid increase both in the number of units in action and in the number of impulses from each unit. Fig. 35 is an example, made from a cat under evipan with a fine wire electrode recording from a single unit. The motor cortex on the left side was stimulated 30 times per sec. by break shocks not strong enough to be painful to the tongue. The first strip (Fig. 35 A) was taken 4 sec. after the beginning of stimulation. Only three of the shocks

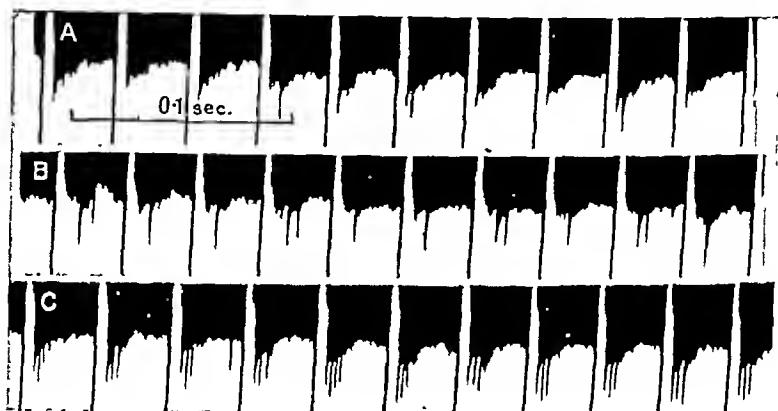


Fig. 35. Single unit record from pyramidal decussation showing increase in response with repeated stimulation of motor cortex. Cat under evipan. Stimuli (30 per sec.) shown by large artefacts. A. 4 sec. after beginning of stimulation. Occasional impulses. B. 5 sec. after beginning of stimulation. Single or double impulses to each shock. C. 8 sec. after beginning of stimulation. 3-4 impulses to each shock. Movement begins.

produce a distinct impulse, though it is quite possible that there was a more regular discharge in other units of the pyramidal tract. At 5 sec. from the start (Fig. 35 B) every shock is followed by an impulse or a pair of impulses 4-6 m.sec. apart. In the third strip, C, 8 sec. from the start, facilitation is well advanced and each shock now produces a group of three or four impulses, spaced at intervals as short as 1.75 m.sec.; it was at this time that a gradual extension of the right forelimb was first noticed.

In many preparations, as in this, the movement does not begin at once and there is a fairly close correspondence between its first appearance and that of the multiple impulses following each shock. In fact we have an association between movement and high-frequency discharge like

several hundred a second, followed by a longer period in which the impulses become grouped into high-frequency outbursts. As soon as this occurs the sustained contraction of the muscles gives place to clonic movements in phase with the outbursts. The records in Fig. 38 show the development of the after-discharge in this way. The outbursts are usually shorter than those produced by convulsant drugs, but the arrangement and frequency of the impulses is much the same. The outbursts and the corresponding movements continue for a few seconds at increasing intervals, the impulse frequency remaining high but the number in each outburst becoming fewer and fewer. When the discharge has finally ceased there is a period of complete inactivity in the pyramidal tract and in the motor area, succeeded eventually by the return of the usual spontaneous discharge.

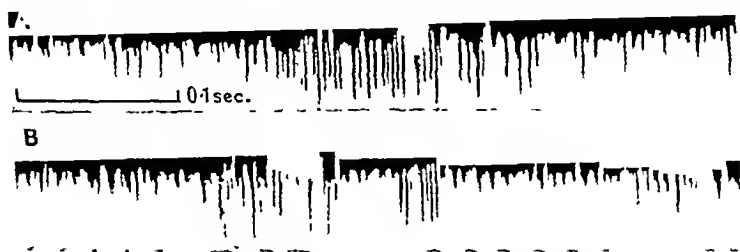


Fig. 39. Monkey (rhesus) under dial. After-discharge in pyramidal decussation following electrical stimulation of motor area (arm) leading to movement. Clonic movement during after-discharge. A. Record 10 sec. after stimuli cease. B. Record 34 sec. later. Fully developed high-frequency outbursts, 700 per sec.

It has been pointed out that the repetitive outbursts set up by convulsant drugs might be due to the poisoned nerve cells reverting more slowly to the inactive state, or to a slowing of the mechanism of adaptation, so that each period of excitation gives a series of impulses instead of a single one. If this is correct, we must suppose that repeated electrical stimulation produces a similar failure to revert immediately to the inactive state. There is no evidence to show how such a change might be produced, but electrical stimulation could certainly lead to transient injury, e.g. by polarization at metallic electrodes.

One experiment involving electrical stimulation was made on a monkey under dial. The results were in general agreement with those from the cat. Movements occurred when the stimuli gave multiple impulses and there was an after-discharge of high-frequency outbursts following lengthy stimulation (Fig. 39). The chief difference lay in the

although setting up single impulses in some pyramidal fibres, rarely leads to facilitation: for this to occur at all it seems as though the stimuli must be strong enough to give high frequencies in a few neurones at least.

(m) *The after-discharge*

Evidence of the same kind comes from the results of prolonged stimulation. If this is continued after the multiple outbursts have appeared a stage is often reached in which they occur at variable intervals and with variable frequencies (cf. Fig. 37). A similar irregularity has

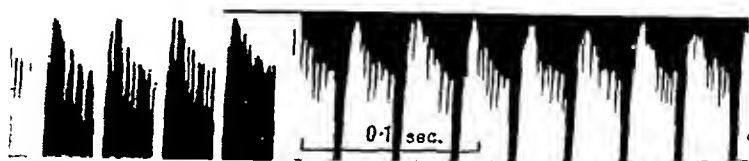


Fig. 37. High-frequency discharges occurring irregularly when facilitation is well advanced. Cat under evipan (same animal as in Fig. 36). Stimulation for 7 sec. at four times threshold strength. Movement of forelimb.

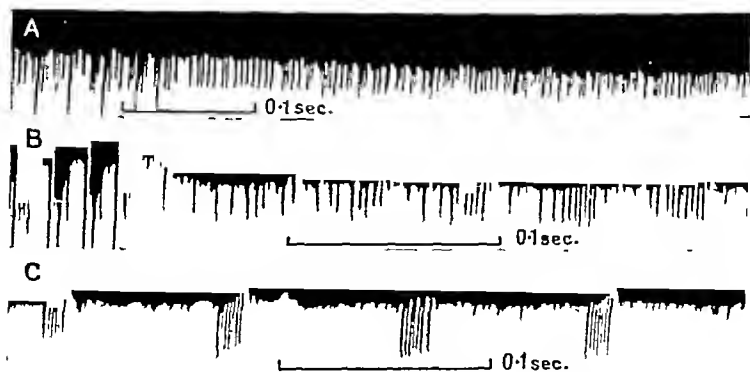


Fig. 38. Development of after-discharge following stimulation of motor cortex. A. Cat under chloralose. Irregular discharges when stimuli cease, changing into short high-frequency outbursts. B. Cat under dial. Same result as A. C. Cat under evipan (same animal as Fig. 35). Fully developed outbursts 1 sec. after stimuli cease. Frequency rises to 950 per sec. All three records accompanied by movement.

been shown in the potential waves of the rabbit's cortex when a high degree of facilitation has been reached [Adrian, 1936], for the waves may then start from various points at a distance from the original focus. When the stimulation is brought to an end there is usually a brief period in which there is a continuous or irregular discharge at a frequency of

several hundred a second, followed by a longer period in which the impulses become grouped into high-frequency outbursts. As soon as this occurs the sustained contraction of the muscles gives place to clonic movements in phase with the outbursts. The records in Fig. 38 show the development of the after-discharge in this way. The outbursts are usually shorter than those produced by convulsant drugs, but the arrangement and frequency of the impulses is much the same. The outbursts and the corresponding movements continue for a few seconds at increasing intervals, the impulse frequency remaining high but the number in each outburst becoming fewer and fewer. When the discharge has finally ceased there is a period of complete inactivity in the pyramidal tract and in the motor area, succeeded eventually by the return of the usual spontaneous discharge.

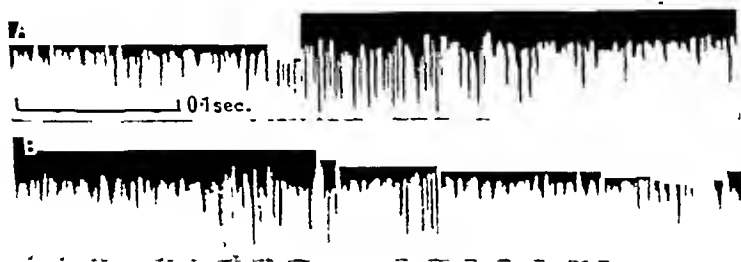


Fig. 39. Monkey (rhesus) under dial. After-discharge in pyramidal decussation following electrical stimulation of motor area (arm) leading to movement. Clonic movement during after-discharge. A. Record 10 sec. after stimuli cease. B. Record 34 sec. later. Fully developed high-frequency outbursts, 700 per sec.

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One experiment involving electrical stimulation was made on a monkey under dial. The results were in general agreement with those from the cat. Movements occurred when the stimuli gave multiple impulses and there was an after-discharge of high-frequency outbursts following lengthy stimulation (Fig. 39). The chief difference lay in the

fact that with the much larger motor area there is a much greater probability that the Betz cells which are stimulated will not be those supplying the particular group of pyramidal fibres under the recording electrode. Thus localized movements could often be produced although no impulses appeared in the record. On the other hand, movements were always produced when the pyramidal fibres showed a multiple discharge.

(n) *Extinction*

These results may have some bearing on the phenomena of "extinction" studied recently by Dusser de Barenne and McCulloch [1937]. They find that in monkeys under dial stimulation of the motor cortex leads after the initial period of facilitation to a longer period of extinction in which the motor response is reduced or absent. They have shown that the extinction is a cortical phenomenon localized to the area stimulated, and that it is increased by raising the strength of the previous stimulation. At first sight it would seem that extinction is merely the result of exhaustion of the stimulated region, but Dusser de Barenne rejects this explanation because (a) extinction is found after weak and even after subliminal stimulation, and (b) it does not appear immediately after the end of the stimulation but is preceded by the state of facilitation.

As all but one of our stimulation experiments have been on cats and as we have not copied the technique used by Dusser de Barenne, we have some hesitation in comparing our results with his. Yet the argument that extinction is not merely the result of exhaustion seems to be considerably weakened by our findings. We have seen that stimulation which produces only a small movement or no movement at all may none the less excite the Betz cells and send impulses down the pyramidal tract. In fact, if the anaesthesia is deep no movement will occur until the pyramidal discharge has reached considerable intensity. Thus stimulation which is weak or subliminal, judged by its motor effects, may be quite capable of causing activity and consequent fatigue in the motor cortex. The fact that extinction does not appear immediately after the end of the stimulation might be due to the development of an after-discharge not great enough to cause movement but capable of increasing the excitability of the surrounding area. The failure of such an after-discharge would then be followed by a general fall of excitability.

In observations on single units of the pyramidal tract "extinction", i.e. complete failure of activity lasting several seconds, has followed stimulation of the cortex only when the stimulation succeeded in producing an impulse discharge in the unit. Extinction in this case is the

sequel of activity and does not appear without it. There is, in fact, a considerable likeness between the period of complete inactivity following an after-discharge and the pauses between the outbursts due to convulsive drugs. With strychnine or picrotoxin on the cortex the high-frequency outbursts continue for many minutes, but the usual arrangement (under dial) is for groups of three or four outbursts to occur close together with inactive periods of 5-10 sec. between one group and the next. The intense activity developed in the high-frequency discharge seems to necessitate a period of complete inactivity as a sequel; the main difference is that with the convulsive drug recovery leads to further outbursts, whereas with electrical stimulation the conditions favouring outbursts disappear soon after stimulation has ceased: thus recovery from the inactive period leads to a return of the usual spontaneous discharge.

DISCUSSION

The chief questions for discussion concern the application of these results to the normal animal. The records have all been obtained from the anaesthetized brain and might have little relation to what goes on in normal life. To what extent therefore are the conclusions likely to be true of the brain without an anaesthetic?

The main conclusions may be grouped under three heads: the first dealing with the relation between the cortical waves and the pyramidal discharge, the second with the variation in the frequency of the impulses in single units and the third with the repetitive outbursts.

In regard to the first we have the result that in the anaesthetized brain, whenever the cortical potential rhythm is well marked there is a corresponding rhythmic discharge in the units of the pyramidal tract. In the unanaesthetized (human) brain there may be well-marked potential waves. It is true that they are usually more sinusoidal in form than those from the brain under dial, and that it is unsafe to generalize from the cat's motor area to the whole of the human cortex; yet in so far as there is a definite rhythm in the surface potentials, we may take it as highly probable that there is a similar rhythm of discharge from the deeper cells, whatever the region concerned and whether there is an anaesthetic or not. In fact we may conclude, for example, that the presence of Berger's α waves implies a discharge of 10 impulses a second in the axons leaving the deeper cell layers.

Under the second head we have to decide whether the pyramidal discharges in the intact animal are likely to differ in impulse frequency,

etc., from those we have recorded. One of the outstanding effects of dial and chloralose is to make the cortex very easily fatigued. In the unanaesthetized brain, therefore, it is probable that impulse discharges can be maintained at the higher frequencies for much longer periods. But fortunately differences of this order can scarcely affect the main conclusion, that the frequency of discharge from the Betz cells varies widely with the level of excitation of the cortex. If the frequency can vary from 5 to 100 per sec. in chloralose, evipan or MgSO_4 anaesthesia, it is most unlikely that the range would be narrower with no anaesthetic. It must follow that the neurones of the motor cortex, when acting under normal conditions, are not restricted to particular rhythms of discharge but can vary their rate like sensory endings and the motor nerve cells of the spinal cord, and if the Betz cells can do so it is highly probable that cells in other parts of the cortex can also discharge at varying frequencies. There was already some evidence for this view and the fixed rhythms of the electro-encephalogram need not be taken as an argument against it [cf. Adrian, 1937].

Under this head we have also to decide whether the pyramidal discharge is likely to have a greater effect on the spinal cord in the unanaesthetized animal, for when there is an anaesthetic a low-frequency discharge of impulses from the motor cortex seems incapable of activating the motor neurones of the spinal cord, though movements may be produced by higher frequencies. Dial in large doses and chloroform and ether certainly reduce the excitability of the spinal cord as well as that of the brain, and the failure of the low-frequency spontaneous discharge may be due in part to this. Yet there is reason to believe that a low frequency would be subliminal for the cord even in the unanaesthetized animal. With chloralose, for instance, the spinal cord may be very little affected by a dose adequate to give complete anaesthesia, for the animals were found to develop good decerebrate rigidity when the brain stem was transected at the end of the experiment. In these animals the pyramidal discharge induced by a sensory stimulus failed to cause movement when the frequency was low (20 per sec. or less), but did so when the frequency was high, even though the discharge was very brief. If it is true that the excitability of the cord was not reduced by the anaesthetic, it follows that low-frequency discharges would be subliminal for the normal animal as well. This does not mean that they would have no effect at all, for subliminal discharges from the motor cortex may very well play an important role in determining the activities of the spinal neurones [cf. Liddell, 1934, 1938].

It is probable, therefore, that the pyramidal discharge must always attain a certain minimal intensity before it becomes supraliminal and causes a movement. To attain that intensity a certain number of impulses must reach a given motor nerve cell within a given time, though it may not be necessary for all of them to arrive by the same pathway. Thus in the normal animal a discharge with a low frequency in each unit might become supraliminal because a large number of units were in action, or because the discharge was continued for long periods, whereas in the anaesthetized animal a discharge at the same frequency would fail because it occurred in few units or was not maintained.

This possible difference is mentioned to emphasize a point which we are in some danger of neglecting. In regions where nervous pathways converge the effectiveness of a discharge will always depend on three factors: (a) the number of converging fibres in action, (b) the frequency of impulses in each fibre, and (c) the duration of the discharge.¹ Since we have been concerned mainly with single units we may have laid too much emphasis on the one factor of impulse frequency. On the whole, however, the number of units in action and the frequency in each unit would not be likely to vary independently. They might do so in the case, for example, of sensory discharges arising from discrete points scattered over a wide area, but in the motor cortex and throughout the central nervous system the level of excitation of one neurone could scarcely increase without some parallel change in its immediate neighbours.

These considerations apply to the high-frequency outbursts as well as to the discharges at lower frequency. Although the convulsive movements due to strychnine or picrotoxin on the cortex are always associated with high-frequency outbursts in some units, yet in other units there are only two or three impulses, at longer intervals, for each outburst. It has been argued that the convulsive movements are due to the high-frequency outbursts because these are specially capable of overcoming the synaptic resistances of the cord: but the effect might also depend on the arrival of a large number of impulses almost simultaneously from a large number of pyramidal fibres converging on to the same synaptic area. The fact is that a focus of abnormal excitation in the motor cortex might well lead to high-frequency outbursts from the Betz cells most directly affected and to outbursts of two or three impulses from many others, and if the

¹ Another factor which may sometimes be important is the timing of impulses in the different fibres, for synchronized volleys might be more effective than independent firing.

summed effect on the spinal synapses is great enough the motor nerve cells will be activated. We cannot say that the activation must be due specifically to the concentrated discharge in some units or to the large number of units giving a few apiece, for we cannot have the one without the other.

This raises the question whether high-frequency discharges are ever likely to occur in the normal animal. Here the answer is certainly "No" as far as the fully developed outbursts are concerned. They would be just as likely, perhaps more likely, to appear in the unanaesthetized brain as in the anaesthetized, but only as the result of convulsive drugs, prolonged stimulation or during epileptiform attacks, for they are essentially the result of abnormal conditions. On the other hand, impulses in groups of two or three close together might sometimes occur in the pyramidal fibres of the normal animal as they do in animals under dial. On general grounds, however, a grouping of this kind would be more likely in conditions in which the cortical waves rise abruptly, as they do under dial, and in the experiments with evipan and MgSO_4 anaesthesia deliberate movements have been associated with a pyramidal discharge made up of impulses spaced at regular intervals (as in Fig. 17). On the whole, therefore, there is no reason to suppose that double or treble impulse groups occur commonly during normal movement.

But although repetitive discharges would not be expected in the normal animal, it is none the less important that they can be found when conditions are abnormal. It follows that the pyramidal discharges and no doubt other discharges from the cortex, have unexpected powers of variation, for a single pulse of activity may lead to a discharge varying from a single impulse to an outburst of twenty or more at a very high frequency. Thus with convulsant drugs the discharge may become intense enough to penetrate the most resistant synaptic barriers, and even in normal conditions it may be able to signal the contour as well as the frequency of the waves of excitation in the cortex.

The foregoing account has many points of contact with other investigations of cortical activity, notably with the work of Renshaw *et al.* [1938], Forbes and Morison [1939], Marshall *et al.* [1937], and Bremer [1938] on the effects of sensory stimulation, and of Jung & Kornmüller [1938] on the potential changes in the cortex and basal ganglia. But discussion of these points must be postponed until we know more about the exact relation between the impulses in a unit of the pyramidal tract and the potential changes due to the same unit in the motor cortex. It is unlikely that the evidence can ever be as definite as that obtained by

Matthews and his colleagues from the spinal cord, yet some evidence should be obtainable for conditions in the cortex have turned out less complex than was to be feared.

CONCLUSIONS

The activity of the motor cortex has been studied by recording impulses in the fibres of the pyramidal decussation as well as potential waves in the cortex. The following results have been obtained:

(1) In the anaesthetized cat, except in the deepest anaesthesia, there is a persistent spontaneous activity in the motor system and the discharge of impulses in the pyramidal fibres corresponds closely with the potential waves in the motor area (sigmoid gyrus). With dial the cortical waves occur in groups at 7-10 per sec. and the impulses in the pyramidal tract are grouped in the same way. With ether the frequency of the cortical waves often rises to 40-60 per sec. and the pyramidal discharge has the same frequency.

(2) With a fine wire electrode the action potentials can be recorded in single conducting units of the pyramidal tract (single axon or groups acting in unison). It is found that the discharge in the single unit agrees in the same way with the cortical potentials, the impulses occurring at 7-10 per sec. with dial and 40-60 per sec. with ether. Sometimes in place of a single impulse there is a group of two or three very closely spaced.

(3) In unfavourable conditions (low blood pressure, etc.) the pyramidal discharge may fail although surface potential waves can still be recorded from the motor area. A temporary dissociation can be produced by a period of cerebral anaemia.

(4) In lighter anaesthesia sensory stimulation often causes an increase in the pyramidal discharge, the frequency in single units rising to 50-150 per sec. The discharge is sometimes accompanied by movement of a limb. The frequency varies over a wide range and there is no tendency to one or more fixed values.

(5) With chloralose an abrupt stimulus will produce a convulsive movement. The reaction involves the motor cortex and is due to a sudden discharge of impulses in the pyramidal fibres. It is often followed by a longer series of impulses with a frequency range which varies with the intensity of the stimulus from 10 to 200 per sec.

(6) Records like those from the pyramidal decussation can be made from the white matter of the motor cortex, but the origin of the impulses is less certain as the white matter contains fibres from other parts of the brain.

(7) Low-frequency discharges in the pyramidal fibres produce no obvious motor effects (movement or change of tone). To become supraliminal for the motor nerve cells of the spinal cord, the discharge must exceed a threshold intensity (frequency in each unit and number of converging units in action) whether the cord is affected by the anaesthetic or not.

(8) When convulsant drugs (strychnine, picrotoxin, etc.) are injected or applied locally to the motor cortex, the pyramidal discharge takes the form of a series of high-frequency outbursts, the impulses in each unit occurring in groups of 10-80 at frequencies of 500 to 1000 per sec.

(9) The outbursts seem to be made up of prolonged repetitive discharges from the Betz cells, due to an abnormally slow decline in each period of activity. Some of the action potentials are due to several neurones working in phase. It is unlikely that the outburst is due to a wave of excitation spreading over a series of Betz cells and making each discharge one impulse.

(10) With convulsant drugs sensory stimulation produces the high-frequency outbursts before they begin to occur spontaneously, and when they occur spontaneously they can be produced at shorter intervals by stimulation.

(11) High-frequency outbursts in the pyramidal fibres, when fully developed, produce convulsive movements except in very deep anaesthesia. This type of discharge seems to be specially capable of overcoming the synaptic resistances in the spinal cord.

(12) Electrical stimulation of the motor cortex may produce movement because each shock sets up a brief high-frequency discharge. The number of impulses to a single shock increases in each unit as facilitation develops, and there is an increase in the number of units in action. Weak stimuli at lower frequencies (30 per sec. or less) may send single impulses down the pyramidal fibres, but the discharge is subliminal for the cord and no movement results.

(13) Following electrical stimulation there may be an after-discharge of high-frequency outbursts, associated with clonic movements if the anaesthesia is not too deep. This is succeeded by a period of complete inactivity. It is suggested that the phenomena of "extinction" may be due to this period of exhaustion following activity.

(14) The foregoing results have been obtained from the anaesthetized brain. There is reason to think that in the normal animal the pyramidal discharges could be maintained at high frequencies for longer times, but that they would show the same wide variation in frequency according to the degree of excitation of the cortex.

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THE EFFECT OF ANTERIOR PITUITARY EXTRACTS ON THE INSULIN CONTENT OF THE PANCREAS

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It has been reported previously [Campbell & Best, 1938] that the insulin content of the pancreas of a dog, in which permanent diabetes had been produced by injections of anterior pituitary extract, was very much lower than the normal value. The present study deals with this point in more detail and also with the effect of anterior pituitary extracts on the insulin content of the pancreas during the stage of transient diabetes. In one phase of this condition which we have studied, the insulin content falls to a very low level, but recovery takes place when the administration of the extract is discontinued. Histological studies on the tissues from this series of animals are in progress and the results will be reported elsewhere by Prof. A. W. Ham and one of us (R. E. H.).

METHODS

Male dogs with normal fasting blood sugars and weighing approximately 10 kg. were used as test animals. Each morning, before food was given, blood for sugar estimations was taken. The animals were then injected subcutaneously with the anterior pituitary extract and allowed to exercise. Food was given in the morning, 2 hr. after the injection, and in the afternoon at 4.00 p.m. Each meal consisted of 200 g. minced lean beef and 25 g. sucrose.

The extract of anterior pituitary gland used in these experiments was prepared as described by Campbell & Keenan [1939] and was used immediately. The anterior portions of the pituitary glands of cattle were frozen in carbon dioxide snow as soon as possible after removal from the animals. The minced glands (e.g. 100 g.) were extracted with 300 c.c. 10% sodium chloride and after 1 hr. again extracted with 100 c.c. of the

same solution. The viscous, supernatant mixture was dialysed against cold, running water until free from chlorides. The precipitate was then centrifuged, washed with cold carbon-dioxide-free distilled water at pH 5.5 and extracted with 5 % sodium chloride at pH 7. The opalescent solution was again dialysed and the precipitate extracted with 5 % sodium chloride at pH 7. (More recently the salt concentrations have been somewhat reduced.) The solution was diluted to contain about 25 mg. organic solids per c.c. and used for subcutaneous injections after dilution with 2 vols. water. During the preparation of the extract the temperature of the mixtures was kept below 10° C.

The amount of anterior pituitary extract injected varied somewhat from day to day, depending upon the condition of the animal, but on the average 24 mg. organic solids per kg. of body weight were given at each injection. As a rule, determinations of the fasting blood-sugar level were made at 24-hr. intervals. Blood and urine sugars were determined by a modification of the Shaffer-Hartmann method, and urinary ketones by the method of Van Slyke [1917]. The pancreas was removed under nembutal anaesthesia and the insulin content determined as described by Best, Haist & Ridout [1939].

RESULTS

Transient diabetes

During the stage of transient diabetes, when the extract was still being given, there was a progressive rise in blood sugar. When the administration of the extract was discontinued the fasting blood sugar rapidly fell to normal values. This is indicated in Table I which shows the rise in blood sugar accompanying seven daily injections of anterior pituitary extract and the fall which occurs when the injections are stopped. The number of animals becomes smaller because one was sacrificed each time a pancreas was obtained for insulin assay. In all the animals receiving four or more injections of the extract glucosuria was present. In these animals, the incidence and extent of ketonuria were variable.

Table II shows the insulin content of the pancreas of six normal animals, as well as the values obtained during the 7-day injection period and the subsequent recovery phase. The pancreas of one animal was taken 8 hr. after the first injection of anterior pituitary extract; the next, 1 day after the injections started; the next, 2 days after, and so on. Seven¹ injections were given in all and the insulin content of the pancreas

¹ One animal given injections for 11 days showed an insulin content of 0.3 units per g. pancreas.

TABLE I. Blood-sugar values following the daily administration of anterior pituitary extract to normal dogs. First injection at time 0. Seven daily injections given

		Blood sugar mg. %												
Dog no.	...	140	139	143	117	121	124	173	176	177	163	167	145	Av.
No. of daily injections	...	1	1	2	4	7	7	7	7	7	7	7	7	
Injection period														
0		91	112	88	95	104	109	97	90	74	99	113	106	98
8 hr.		113	—	—	—	—	—	—	—	—	—	—	—	113
1 day		—	149	106	103	119	130	130	120	112	193	154	123	131
2 days		—	—	119	116	132	144	—	—	—	160	198	145	145
3	"	—	—	—	124	130	139	210	180	195	186	213	—	172
4	"	—	—	—	110	—	—	283	268	257	252	272	—	240
5	"	—	—	—	—	196	256	—	—	—	219	279	226	235
6	"	—	—	—	—	195	206	270	240	209	345	345	—	259
Recovery period														
7 days		—	—	—	—	182	198	195	227	200	268	248	180	212
8	"	—	—	—	—	—	—	—	104	90	90	94	—	94
9	"	—	—	—	—	—	—	—	—	65	—	—	—	65
10	"	—	—	—	—	—	—	—	—	—	91	99	—	95
14	"	—	—	—	—	—	—	—	—	—	—	109	—	109
33	"	—	—	—	—	—	—	—	—	—	—	—	112	112

TABLE II. The effect of anterior pituitary extract on the insulin content of pancreas

		No. of daily injections of A.P.E.	Time of pan- createctomy after last injection	Wt. of pancreas g.	Insulin content		
Dog no.	Wt. of dog kg.				Total units	Units per kg. body wt.	Units per g. pancreas
63	13.0	0	0	27.2	105.0	8.06	3.9
87	7.0	0	0	14.5	32.7	4.7	2.3
90	5.5	0	0	16.6	54.8	10.0	3.3
93	8.2	0	0	15.4	46.6	5.7	3.2
96	10.5	0	0	20.1	73.4	7.0	3.6
99	10.7	0	0	20.4	76.9	7.2	3.8
140	17.0	1	8 hr.	40.15	96.5	5.68	2.4
139	13.3	1	1 day	30.58	58.0	4.36	1.9
143	10.6	2	1 "	28.65	54.4	5.14	1.9
117	8.0	4	1 "	30.4	38.6	4.45	1.27
121	10.95	7	1 "	27.49	5.8	0.53	0.21
124	10.2	7	1 "	27.93	6.4	0.63	0.23
173	9.7	7	1 "	21.71	3.9	0.40	0.18
176	9.95	7	2 days	45.82	6.4	0.64	0.14
177	9.0	7	3 "	23.03	35.0	3.89	1.5
163	10.2	7	4 "	25.32	66.0	6.46	2.6
167	9.3	7	8 "	26.29	71.0	7.64	2.7
145	11.3	7	27 "	27.81	100.0	8.88	3.7
125	8.45	11	1 day	22.21	7.3	0.86	0.33

was also determined on the 1st, 2nd, 3rd, 4th, 8th and 27th day following the last injection. It is evident that a very great fall in the insulin content of the pancreas occurs during this 7-day period and that recovery follows the cessation of the injections.

The curves showing the *average sugar values* and the *insulin content* of pancreas determined at a corresponding time, are given in Fig. 1. The graph demonstrates rather strikingly the correlation of these changes under the conditions of this experiment.

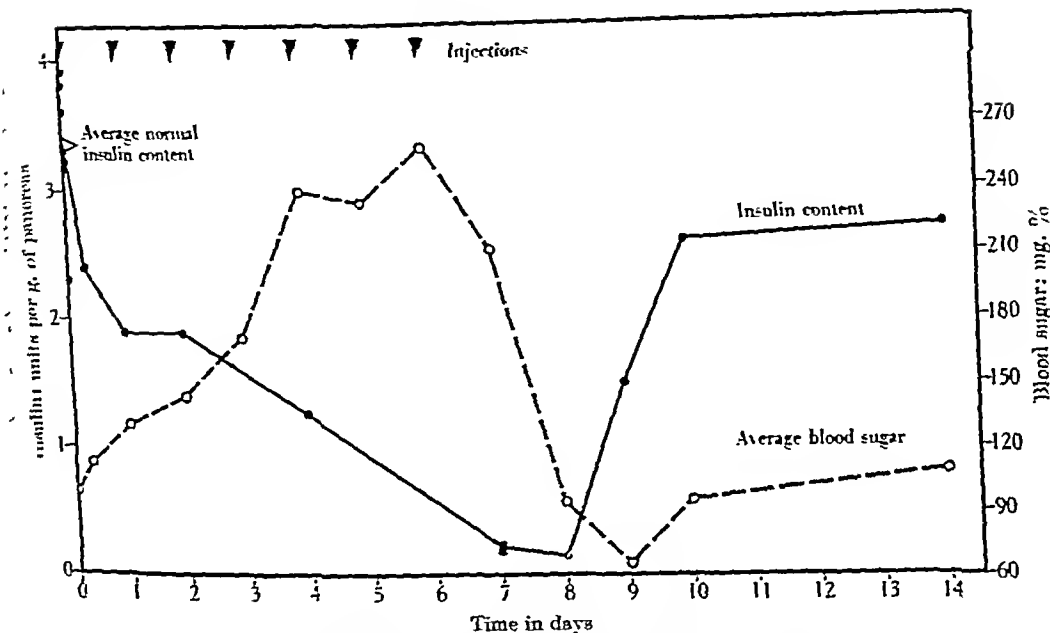


Fig. 1. Effect of anterior pituitary extract on the insulin content of pancreas and average blood-sugar level.

Permanent diabetes

In four dogs injected intraperitoneally with a saline extract of the anterior pituitary gland [for preparation of which, see Young, 1938], permanent diabetes was established after from seventeen to thirty injections. In these permanently diabetic animals the fasting blood sugar ranged from 200 to 400 mg. %. The insulin content of the pancreas of these dogs is included in Table III, with certain relevant data. Dog 3 was thyroidectomized a short time prior to the removal of the pancreas. Dog 4 received two courses of the extract and was hypophysectomized on the 133rd day after the injections were stopped. A gross inspection at autopsy disclosed no pituitary tissue but serial sections of the base of the brain are being made to determine whether or not the removal was complete. It will be evident from this table that the insulin content of the

TABLE III. The insulin content of the pancreas of dogs made permanently diabetic by anterior pituitary extracts

Dog no.	Dog wt. kg.	No. of days on extract	Time after last injection when insulin started days	Time after last injection when pancreatectomy performed, days	Wt. of pancreas g.	Insulin content		
						Total units	Units/kg. body wt.	Units/g. pancreas
1	9.1	17	30	58	19.2	<2.0	<0.22	<0.1
2	8.7	21	59	78	26.2	2.6	0.30	0.1
3	10.4	30	26	192	14.2	3.5	0.34	0.2
4	8.2	30	49	198	19.7	<2.0	<0.24	<0.1

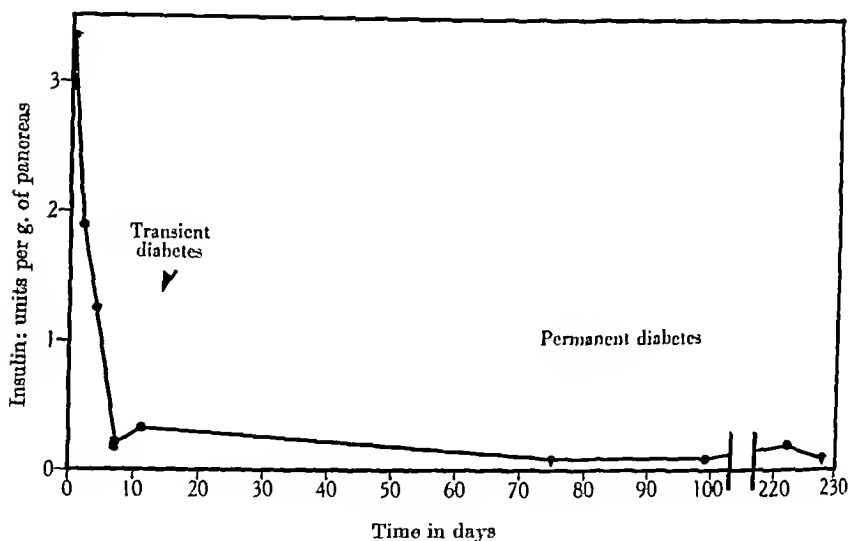


Fig. 2. The effect of anterior pituitary extracts on the insulin content of pancreas.

pancreas of all of these permanently diabetic dogs is very low. Fig. 2 shows the fall in insulin content of pancreas during the course of the injections and also the level in the permanently diabetic animals.

DISCUSSION

The transient diabetes produced by the daily administration of anterior pituitary extract was characterized by a steady increase in blood sugar which reached an average value of about 240 mg. % in 4 days and remained at approximately this level until the 6th day when the last injection was given. The insulin content of the pancreas showed, within 24 hr., a decrease below the average normal value of 3.4 units per g. of pancreas, and reached the low value of 0.2 units per g. at the end of 7 days.

When the administration of the extract was discontinued after the 7th daily injection, the blood sugar fell rapidly to normal values within 48 hr. but the insulin content did not regain its normal level until the 4th day.

It will be observed that the method of expressing the insulin content of pancreas affects considerably the apparent time relationship of the effects of the pituitary extract. Thus, if the values are calculated as units of insulin per g. pancreas, the decrease in insulin content appears to begin earlier than an inspection of the same results expressed as total insulin per kg. animal would indicate. Since the values for the normal pancreases appeared to be much more uniform when calculated on the basis of insulin content per g. tissue, we prefer this method of expression. The ratio of weight of pancreas to body weight is by no means uniform in the dogs which we have studied.

The low insulin content of the pancreas seen in these experiments may result either from an increased liberation of insulin or from a decreased production by the pancreas. Houssay & Foglia [1936] grafted the pancreas of dogs exhibiting the transient pituitary diabetes into the neck of completely depancreatized dogs. They concluded that, in certain cases, their experimental results demonstrated a diminished liberation of insulin from the pancreas of the treated animals. If this is generally true, our results on the insulin content of the pancreas indicate that the production of the hormone may be greatly diminished by the administration of anterior pituitary extract. However, factors other than altered islet function are involved in this transient diabetes since, as previous investigators have shown, (1) injections of the extract initiate or accentuate hyperglycaemia and glucosuria in the depancreatized-hypophysectomized and in the depancreatized dog, and (2) animals in this transient state of diabetes are very resistant to the action of exogenous insulin. These facts indicate that an increase in the liberation of insulin from the pancreas might not be evident, since it could be masked by the antagonistic effects of the anterior pituitary extract exerted on other tissues.

In the condition of permanent diabetes which was produced by seventeen to thirty injections of the extract, extremely low insulin contents of pancreas were noted 58-193 days after the last injection. During most of this time the animals received adequate doses of insulin. No recovery of the insulin content of the pancreas was observed. Campbell, Keenan & Best [1939] observed that total pancreatectomy did not increase the severity of the diabetes, nor the insulin requirements of two of these animals. In permanent diabetes, the very low insulin content of pancreas

is therefore probably accompanied by a decreased output of the hormone. This condition can thus be explained, in large part, on the basis of an interference with the insulin-producing mechanism. In the transient diabetes, the picture is not as clear. As noted above, there may be an increased liberation of insulin which is masked by antagonistic effects, or the first effect may be relatively slight injury which becomes progressively greater when the administration of the extract is continued. In other words, in the transient diabetes, there may prove to be either initial stimulation followed by exhaustion, or progressively increasing damage. The results of the histological studies and of the course of recovery of the insulin content after longer periods of injection will help to settle these points.

SUMMARY

1. The subcutaneous administration of diabetogenic extracts of the anterior pituitary gland produces a prompt and extensive fall in the insulin content of dog's pancreas. For example, as a result of seven daily injections, the insulin content of the pancreas fell from 3.4 to 0.2 units per g.

2. When, after seven daily injections, the extract is discontinued, a rise in insulin content is noted and the normal value is regained within 4 days.

3. The rise and subsequent fall of the fasting blood-sugar values follow a curve the shape of which bears an interesting relationship to that which illustrates the decrease and recovery of the insulin content of pancreas.

4. When permanent diabetes is established in dogs, the insulin content of the pancreas may vary from approximately 0.2 unit per g. (normal value 3.4 units per g.) to amounts too small to estimate.

5. No evidence of recovery of the insulin content has been secured in animals in which permanent diabetes has been observed over long periods (up to 198 days).

We are greatly indebted to our colleagues Dr Jessie H. Ridout and Miss Helen Bell for their invaluable help in the preparation and assay of the insulin-containing extracts.

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AFFERENT IMPULSES FROM THE TEETH DUE TO PRESSURE AND NOXIOUS STIMULATION

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THAT the tooth has a rich sensory endowment is a matter of common observation and clinical experience. Nevertheless, most controlled studies of this subject have been largely histological in emphasis and the present study is concerned with a more physiological approach making use of electro-physiological methods to register the action potentials in the dental nerves of the cat.

HISTORICAL²

The earliest workers. Hunter & Charles Bell [1811], believed that the dentine was insensitive to pain and that its apparent response to stimuli was due to mechanical impulses which were transmitted to the pulp. Clinical observations by Duval [1833] and Thomas Bell [1835] showed that the dentine itself was acutely sensitive to pain. This has been the generally accepted view up to the present time.

Peaselee [1857] mentions that various forms of pressure can be detected and to some extent localized by the teeth. Fränkel [1871] pointed out that this power of localization was due to the nerves of the periodontal membrane and was still present after removal of the pulp. Black [1887] clearly recognized that both the pulp and periodontal nerves were necessary to complete the sensory complex of the tooth. The nerves of the periodontal membrane responded to the slightest pressure, whereas the pulp nerves gave rise to pain whatever the stimulus.

The demonstration by Stewart [1927] that the tactile thresholds of teeth were practically unchanged after extirpation of the pulp strongly suggests that the tactile sensitivity is limited to the periodontal membrane, although it has been maintained that there is a considerable amount of tactile sensibility over and above pressure which is affected by removal of the pulp [Woods, 1914]. Others have suggested that alterations in the calibre of the dental tubules from occlusive stress may be transmitted to the pulp nerves [Sprenkel, 1936].

At the present time, it is most generally accepted that pain is related to the pulp fibres and touch to the periodontal membrane, although pain may arise from this region as well.

¹ George Henry Lewes Student.

² For historical references, see Stewart [1927].

ANATOMICAL

In the cat the tooth pulp is supplied essentially with medullated nerve fibres of small size, between 2 and 10μ in diameter, with few if any unmyelinated fibres [Windle, 1927; Brashear, 1936]. On the other hand, the exact nature of the termination of these fibres is not clear. As the fibres proceed up through the pulp, they progressively lose their myelin sheaths and pass into the region of odontoblast cells and, according to some [Tiegs, 1932, 1938] may end in that region in special endings or, according to others [Sealey, 1932; Lewinsky & Stewart, 1936; v. d. Sprenkel, 1936], may continue into the dentine as fine fibres for varying distances.

The nerve supply to the periodontal membrane, on the other hand, is more heterogeneous including unmyelinated as well as myelinated fibres up to 14μ in diameter [Windle, 1927; Brashear, 1936]. These are derived from the apical nerves, as well as from various bundles penetrating the numerous foramina in the alveolar bone [Lewinsky & Stewart, 1937]. These latter divide into two fasciculi, one of which runs toward the apex, the other toward the gingival margin, and consist of two types of fibre, thick ones confined to the peripheral part of the membrane, which in the cat have specialized spindle-like terminations formed by the fibre becoming twisted like a spiral spring, and finer ones which pass to the deeper parts of the periodontal membrane and there break up into fine arborizations without terminal organs.

APPARATUS AND PROCEDURE

The recording apparatus consisted of a resistance capacity-coupled amplifier, loud-speaker, and Matthews oscillograph arranged for visual scanning as well as photography.

Nerves supplying the incisor, canine and premolar teeth of the upper jaw were obtained in cats anaesthetized under dial after first removing the eye and then locating the dental branches of the maxillary division of the trigeminal nerve as they cross the floor of the orbit. The head was rigidly held by a clamp which engaged the upper jaw by the infra-orbital margin of the opposite maxilla superiorly and the opposite molar teeth inferiorly. This clamp was applied directly without reflexion of the skin.

The whole animal was placed in a heated box fitted with a sliding glass front. Temperature and humidity could be independently controlled and maintained to keep the nerve in good condition. Silver-silver chloride wick electrodes were used.

For threshold measurements of pressure, a series of bristles was made, calibrated on a balance to bend at the following values:

Bristle	g.
1	5-7
2	2-3
3	1
4	0.5
5	0.25

Heavier weights were applied by means of a simple lever, one end of which could be fastened to the tooth by plasticine, while the appropriate weights were attached to the other. This could be arranged to work either in the vertical or horizontal planes.

EFFECTS OF PRESSURE

When the intact tooth is touched with an insulated rod, there is a marked discharge of nerve impulses. At the moment of contact there is an initial high voltage spike (250-600 μ V.) which is followed by a steady



Fig. 1. Response to pressure showing the initial high-voltage spikes produced at the moment of contact, followed by the low-voltage asynchronous discharge. Both records from the same preparation. A. Calibration, 40 μ V. Note that the initial spikes are off the record. B. Calibration, 400 μ V. Time 0.1 sec.



Fig. 2. Response of the whole nerve to maintained pressure (100 g.) on the canine tooth. The four strips of record reading from the left illustrate the response at the beginning, after 15, 45 and 180 sec. of stimulation respectively. Time 0.05 sec.

asynchronous discharge of much lower voltage (15-40 μ V.) as long as the pressure is maintained (Fig. 1). Over a period of time this steady

discharge shows a gradual diminution in potential magnitude and in complexity due both to a decrease in the frequency of response in some fibres and to a cessation of activity in others (Fig. 2).

Touching neighbouring teeth and regions of the gum and lower jaw whose nerves are not on the electrodes shows that this initial response is not an artefact resulting from the mere contact, but is nervous in origin and represents presumably a single synchronous volley in the nerve. Single taps give rise to the large spikes only.

When the stimulus is removed after maintained pressure, there is an immediate cessation of the response. In two cases, after the pressure had been maintained for a considerable period of time, there was a slight discharge of a few fibres for a short period after its removal. Only once did a single fibre (judging from the potential record) of the large number active during normal tactile stimulation give rise to a short series of impulses upon removal of the stimulus.

Many, if not most, of the endings responsive to tactile or pressure stimulation are located in the periodontal membrane and receive their nerve supply through alveolar bone, since the response diminished little, if at all, after removal of the pulp and destruction of the nerves in the apical canal by a cautery. After severe fracture of the tooth and removal of all but a small portion of the root, pressure on this remaining fragment still gave rise to a vigorous response. In other experiments, where single fibres or only a few fibres were obtained, removal of the pulp and apical canal nerves by pulp canal cleaners did not affect the response to pressure.

Determinations of thresholds were made with the series of graded hairs. In most adult cats, the threshold value for the canine tooth was 2-3 g. In one very young animal a pressure of 0.5 g. was found to be effective, whereas for all cases 0.25 g. was found to be supraliminal for the skin of the nose or the mucous membrane of the gums and tongue. In the tooth, the threshold bristle often stimulated only a few and in some cases only one fibre. Different fibres in the same and different preparations gave varying adaptation times (i.e. time to cessation of the response with a maintained pressure) with the same stimulus. These differences in adaptation time were also apparent for supraliminal stimuli in experiments in which single fibres were obtained by dissection. With a 100 g. stimulus some endings gave only a few impulses (five or six or less), whereas others continued to discharge for 5 min. or longer. No attempt was made to follow the response for longer than 5 min.

The response of any one ending to pressure consists of a regularly spaced train of impulses (Fig. 5 A). With greater pressures the frequency

of response is higher and usually adaptation time is longer (Fig. 3). The relationship between the frequency of response and the stimulus over the

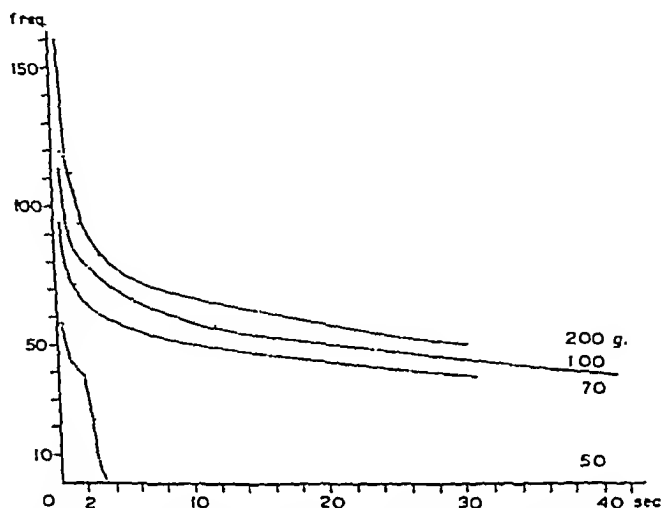


Fig. 3. Showing the frequency of response in the same fibre for different pressures.

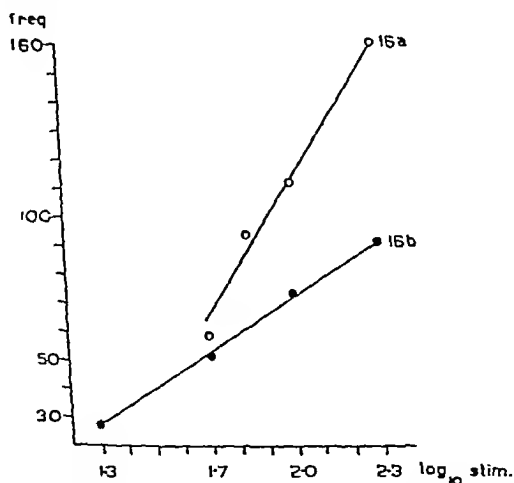


Fig. 4. The relation between frequency of response and log of the stimulus for two different experiments. 16 a is based on the frequency during the first sec. of the response shown in Fig. 3. 16 b is a similar plot from another experiment.

limited range used in these experiments (20-200 g.) is approximately logarithmic as indicated in Fig. 4. Since in both cases only four points

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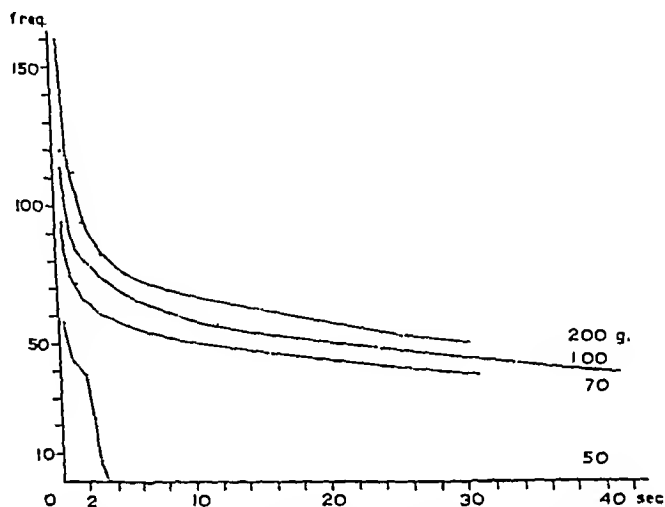


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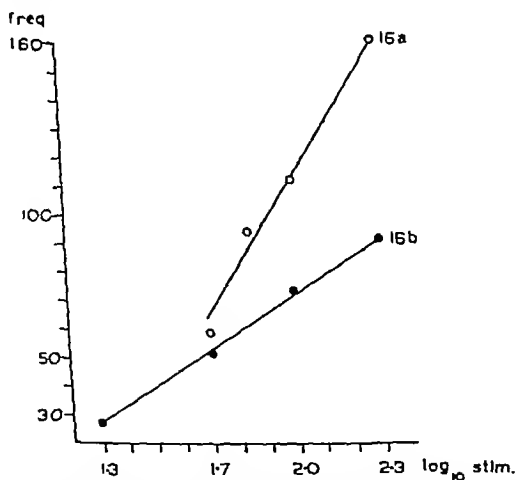


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were obtained and since the experiments were not especially designed to test this aspect of the response, the results are included merely to show that they are in keeping with the properties of other afferent endings [Matthews, 1931; Hartline & Graham, 1932].

When the full nerve trunk was placed on the electrodes, it was noted that pressures applied against any surface of the tooth elicited responses of about the same magnitude. With single fibres, however, it was found that pressures against only one surface were effective for that particular fibre. Thus, in Fig. 5, the upper record illustrates the response to a stimulus applied to the canine tooth in the cephalo-caudal direction. The second record shows that the opposite direction does not stimulate the same ending, but that this direction is adequate for several other fibres. From



Fig. 5. Showing different fibres active with pressures applied in opposite directions. A. Cephalo-caudal direction. One fibre. B. Caudo-cephalic direction. Several other fibres. Time 0.2 sec.

the maximal position, there is a decrease in stimulating efficiency until a position of about 90° on either side is reached where the stimulus is no longer effective for that particular fibre. This suggests that, since most of the endings are in the periodontal membrane, only one type of deformation of the ending, as the tooth moves slightly in the alveolus, is effective. From the case reported above, where a fragment of the tooth was stimulated by pressure, it might be argued that pressure only and not tension is the adequate stimulus, although it must be remembered that conditions were abnormal.

The nerve branches to the teeth may also contain fibres supplying the adjacent gums and even lips. These, too, may be activated individually by localized stimulation with a graded bristle, and seem to resemble

physiologically those endings found in the periodontal membrane, showing on the whole slow adaptation and individual variations in time to complete adaptation. In some cases a spontaneously discharging ending may be encountered which can be made to stop after maintained pressure over the sensitive area. When the stimulus is released, there is a cessation of all response for a time, followed by a gradual return to the level of the previous resting discharge. This has also been observed occasionally in a tooth and is reminiscent of the same behaviour reported for muscle endings [Adrian & Zotterman, 1926 *a*; Matthews, 1933]. These slowly adapting pressure endings are also found in the tongue when the afferent impulses from that organ are recorded from the lingual nerve.

In both the periodontal and mucous membrane endings, the frequency of discharge was found to be influenced not only by the final tension or pressure applied, but also by the rate of application of that tension. It was only qualitatively noted that with more rapid changes higher initial frequencies were obtained, and since others [Adrian & Zotterman, 1926 *a, b*; Matthews, 1931] have already carefully elucidated this effect, no further analysis was made. It was noticed, however, that in the tooth, higher frequencies were obtained with sudden applications of pressure than could be obtained from the endings in soft tissues. This is probably due to the damping effect of the soft tissues which prevent sudden changes from being directly applied to the end-organ in question. The highest initial frequency obtained to such sudden changes in pressure was 1200 per sec., although this high rate of discharge lasted for only a few impulses. This maximum frequency will be discussed later in connexion with the response to a vibratory stimulus.

It is of interest to consider another type of ending found in the tongue characterized by a rapid rate of adaptation. This ending is not stimulated by steady pressure, but only by changes of pressure, and so may respond either upon the application or removal of the stimulus. In this case, the frequency of discharge is also determined by the rate of application of the stimulus. Slow applications may call out impulses at about 25 or less per sec. Sudden deformations caused by tapping the skin with a tactile bristle or other light instrument may call out groups of 5-10 regularly spaced impulses at a frequency of nearly 1000 per sec. This type of ending has never been found in the tooth in these experiments. On the tongue, such endings may be related to a sensitive area of about 5 mm. in diameter as determined by the tactile bristle. The pressure endings of the tongue have a smaller sensitive region on the surface and usually have a higher threshold.

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When the full nerve trunk was placed on the electrodes, it was noted that pressures applied against any surface of the tooth elicited responses of about the same magnitude. With single fibres, however, it was found that pressures against only one surface were effective for that particular fibre. Thus, in Fig. 5, the upper record illustrates the response to a stimulus applied to the canine tooth in the cephalo-caudal direction. The second record shows that the opposite direction does not stimulate the same ending, but that this direction is adequate for several other fibres. From

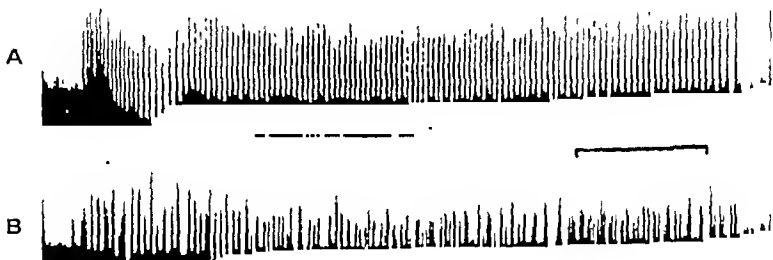


Fig. 5. Showing different fibres active with pressures applied in opposite directions. A. Cephalo-caudal direction. One fibre. B. Caudo-cephalic direction. Several other fibres. Time 0.2 sec.

the maximal position, there is a decrease in stimulating efficiency until a position of about 90° on either side is reached where the stimulus is no longer effective for that particular fibre. This suggests that, since most of the endings are in the periodontal membrane, only one type of deformation of the ending, as the tooth moves slightly in the alveolus, is effective. From the case reported above, where a fragment of the tooth was stimulated by pressure, it might be argued that pressure only and not tension is the adequate stimulus, although it must be remembered that conditions were abnormal.

The nerve branches to the teeth may also contain fibres supplying the adjacent gums and even lips. These, too, may be activated individually by localized stimulation with a graded bristle, and seem to resemble

physiologically those endings found in the periodontal membrane, showing on the whole slow adaptation and individual variations in time to complete adaptation. In some cases a spontaneously discharging ending may be encountered which can be made to stop after maintained pressure over the sensitive area. When the stimulus is released, there is a cessation of all response for a time, followed by a gradual return to the level of the previous resting discharge. This has also been observed occasionally in a tooth and is reminiscent of the same behaviour reported for muscle endings [Adrian & Zotterman, 1926 *a*; Matthews, 1933]. These slowly adapting pressure endings are also found in the tongue when the afferent impulses from that organ are recorded from the lingual nerve.

In both the periodontal and mucous membrane endings, the frequency of discharge was found to be influenced not only by the final tension or pressure applied, but also by the rate of application of that tension. It was only qualitatively noted that with more rapid changes higher initial frequencies were obtained, and since others [Adrian & Zotterman, 1926 *a, b*; Matthews, 1931] have already carefully elucidated this effect, no further analysis was made. It was noticed, however, that in the tooth, higher frequencies were obtained with sudden applications of pressure than could be obtained from the endings in soft tissues. This is probably due to the damping effect of the soft tissues which prevent sudden changes from being directly applied to the end-organ in question. The highest initial frequency obtained to such sudden changes in pressure was 1200 per sec., although this high rate of discharge lasted for only a few impulses. This maximum frequency will be discussed later in connexion with the response to a vibratory stimulus.

It is of interest to consider another type of ending found in the tongue characterized by a rapid rate of adaptation. This ending is not stimulated by steady pressure, but only by changes of pressure, and so may respond either upon the application or removal of the stimulus. In this case, the frequency of discharge is also determined by the rate of application of the stimulus. Slow applications may call out impulses at about 25 or less per sec. Sudden deformations caused by tapping the skin with a tactile bristle or other light instrument may call out groups of 5-10 regularly spaced impulses at a frequency of nearly 1000 per sec. This type of ending has never been found in the tooth in these experiments. On the tongue, such endings may be related to a sensitive area of about 5 mm. in diameter as determined by the tactile bristle. The pressure endings of the tongue have a smaller sensitive region on the surface and usually have a higher threshold.

Discussion

The finding that the periodontal membrane is so richly supplied with nerve endings, the adequate stimulus for which is mere pressure or touch, is in keeping with the observations in man that pulpless teeth retain their tactile sensitivity. In fact, the pressure thresholds of teeth before and after removal of the pulp is little changed as measured by an aesthesiometer. A peripheral basis for the ability to localize fairly accurately the stimulus when applied to the tooth [Stewart, 1927] is provided in the unidirectional sensitivity of the periodontal endings. Furthermore, the fact that such a rich tactile response may be obtained after destruction of the apical nerves agrees with the histological finding that a majority of the nerves to the membrane come from the alveolar plate itself [Lewinsky & Stewart, 1937].

The extreme development of this pressure sensitivity can be related to the reflex control of mastication. In the decerebrate preparation Sherrington [1917] demonstrated that pressure stimulation of the gums bordering the teeth of both the upper and lower jaws, of the teeth themselves, as well as of the front part of the hard palate, caused reflex opening of the tonically closed jaw, which involved a reflex inhibition of the jaw-closing muscles as well as a stimulation of the opener muscles. Faradization of the central end of the severed superior alveolar nerve had a similar result. These effects could also be demonstrated in the anaesthetized animal, and in the present experiments it has often been observed that when the maxillary branch of the trigeminal was cut, there was practically always a very strong and sudden movement of the mandible.

The finding of two definite types of ending in the mucosa of the tongue agrees with the results of Adrian & Zotterman [1926 b] who describe rapidly adapting touch and more slowly adapting pressure endings of the skin. In the present experiments, the fact that the touch endings have a lower threshold suggests that they are not so deeply situated as the pressure endings.

The fact that frequencies up to 1000 per sec. can be elicited in the case of the touch ending and even higher in the case of the tooth ending agrees with the proof by Matthews [1931] that the sensory ending overlaps the nerve fibre with respect to its capacity for response.

RESPONSE TO NOXIOUS STIMULI

Observation of the impulses related to noxious stimuli is complicated by the fact that any manipulation of the tooth causes a pronounced discharge from the pressure endings which quite effectively masks

anything else that may be going on. On the other hand, it is possible to treat the tooth with agents that are definitely related to pain in the human and to avoid this complication.

The best agents for this purpose are hot or cold water. The intact canine tooth can be immersed in the fluid contained in a small beaker or vessel without causing any pressure discharge. Water at room temperature has little effect, but water of 70 or 0° C. will call out a marked discharge of impulses which for the most part are more slowly conducted than those elicited by pressure. Since both cold and hot water produced these impulses and since pressure impulses retained their normal form when the tooth was immersed in ice water, it is not likely that these slower rates of conduction were the result of temperature changes affecting the

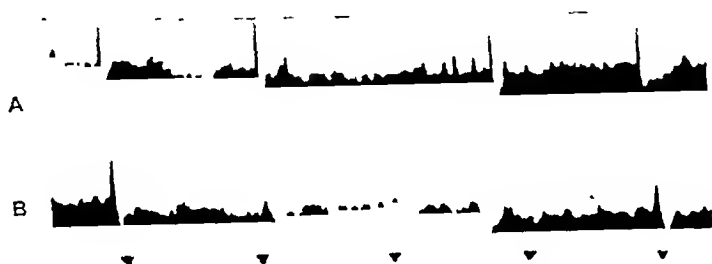


Fig. 6. Comparison of fast and slow impulses. A. Pressure. B. Ice water. Interelectrode distance 4-4 mm. Time 0.01 sec.

nerve. Comparison of the diphasic impulses resulting from both types of stimuli also show that the potentials related to "painful" agents are of longer duration at the electrodes, due to the slower rate of conduction as indicated by the thickness of the potential spike (Fig. 6).

Water of 70° C. seems a more effective stimulus than ice water, for not only are the recognizable impulses called out, but there is also an increased irregularity of the base-line which would indicate that numerous potentials of small magnitude are being produced. For both hot and cold, the impulses continue as long as the stimulus is applied, although there seems to be a diminution in the response as time proceeds.

It might be argued that these stimuli were activating endings specifically sensitive to temperature changes. Injury to the tooth, however, gives rise to impulses of the same type. Clipping off the end of the tooth with a bone forceps also gives rise to a series of slowly conducted impulses which gradually decrease in number with time. It has only been possible to estimate this "adaptation" qualitatively, yet it is quite clear that the

response continues for some minutes after the injury. In certain cases, a large number of small, yet moderately fast impulses have been called up by noxious agents as well. In one case where strong acetic acid was placed on the bared dentine and pulp, there was also a marked discharge which gradually built up and then declined over a period of several minutes. In another case, mere exposure of the dentine elicited a discharge of moderately fast impulses which could be diminished by covering the exposed area with cotton-wool soaked in warm Ringer's solution.

On the whole, however, this response has been disappointingly meagre for a region known to be as sensitive as the tooth. The technical limitations are considered to be responsible for this, since many of the potentials are barely greater than the noise level of the amplifier. In some experiments, where the base-line was particularly bad, no indication of a response to any noxious agent could be detected at all.

Discussion

Estimations of conduction velocity can be made from the form of the diphasic impulses if the interelectrode distance is known. The time of conduction from one electrode to the second is then given by the interval from the beginning of the rising phase of the negative deflexion to the point where this is first affected by the beginning of the opposite phase.

Such estimations possess doubtful accuracy for a number of reasons. Inaccuracies in measurement of the interelectrode distance would result in large errors if this distance is small. Furthermore, in agreement with others, it has been noted that the slowly conducted impulses are often triphasic in form. Adrian [1931] attributes this to axon branching, while Bishop [1934] points out that often, particularly in fibres, there is a large positive after-potential which gives a triphasic form to the recorded impulse. Both factors would vitiate measurements based on the beginning of the second phase.

Analysis of the records themselves is complicated by the difficulty of determining the beginning of the positive phase. Comparison of the fast monophasic spikes with the fast diphasic spikes show that there is a definite break in the rising phase of these potentials. In the slower potentials, however, there seems to be little difference in the spike durations of the monophasic and diphasic impulses, so that the beginning of the second phase is combined with the end of the monophasic potential. Nevertheless, calculations based on the form of the potential have been made. The rates for the fast impulses of 24-60 m./sec. are probably near

the correct value, while those for the slow impulses of 4–21 m./sec. are probably too fast.

Since the errors of measurement seem to be less significant for the fast impulses it seems permissible to make certain correlations by placing them among the slower components of the A group of Erlanger & Gasser [1930]. It seems quite definite that this group is made up of fibres of large size, although the exact relation between velocity and fibre diameter is not yet settled [Erlanger & Gasser, 1937; Douglass, Davenport, Heinbecker & Bishop, 1934]. Histological studies have shown that the nerves to the periodontal membrane in the cat include all sizes but with 20 % fibres between 10 and 14 μ in diameter. In this region large fibres are related to the spindle endings. The fast conduction rates of the pressure impulses are consistent with all these facts.

All the pulp nerves are myelinated, but of smaller size ranging from 2 to 9 μ in diameter with 64 % less than 6 μ in diameter. Noxious stimuli, which presumably activate these fibres, call out impulses of slower conduction rates. This agrees with the results of others that "painful" stimulation gives rise to slowly conducted potentials presumably related to fibres of smaller diameter [Adrian, 1932; Bishop & Heinbecker, 1935; Clark, Hughes & Gasser, 1935; Erlanger & Gasser, 1937; Zotterman, 1939].

The fact that an organ like the tooth, so richly endowed with pain sensitivity, is supplied only with small myelinated fibres is of interest with regard to the relation between fibre types and their function. Histological considerations alone have shown that nerves subserving the same cutaneous qualities may have quite different fibre constitutions with respect to the smaller fibres. The trigeminal, as a whole, has a much smaller unmyelinated component than have the spinal nerves [Windle, 1926]. The pulp nerves of the tooth are essentially myelinated containing many fibres of the B group, yet the quality of the sensation aroused in man by stimulation of that organ can hardly be called "pricking pain". This kind of correlation may hold for any given preparation of the skin [Zotterman, 1939], but cannot be extended generally to other regions of the body. Indeed, this innervation of moderately sized fibres would account for the finding of v. Werz [1932] that the chronaxie of the tooth for pain indicated fast excitabilities of the irritable tissue concerned. Stimulation in this case probably involved direct activation of the medullated pulp fibres.

SUMMARY AND CONCLUSIONS

1. Touch or pressures applied to the intact tooth gave rise to an intense discharge of nerve impulses in the dental nerves.
2. Most of the endings responsible for this discharge are located in the periodontal membrane.
3. Threshold values for the canine tooth are about 2-3 g. using a bristle calibrated to bend at that pressure.
4. The endings to pressure showed great individual differences in adaptation times. Times to complete cessation of the response varied from a fraction of a second to more than 5 min.
5. Individual pressure endings of the periodontal membrane displayed properties agreeing with those elucidated for other afferent endings and are physiologically similar to the pressure endings of the mucous membrane.
6. Force applied only in one general direction stimulates the single ending. It seems that pressure rather than tension is the adequate stimulus for the single ending.
7. Frequencies as high as 1200 per sec. in a single fibre from the tooth have been recorded with very rapid applications of pressure.
8. The touch endings of the mucous membrane (tongue) respond with short bursts of impulses to changes in pressure, but not to steady deformations. Frequencies of nearly 1000 per sec. have been obtained with rapid deformations of the mucous membrane. These endings have a lower threshold than have the pressure endings of the tongue.
9. Noxious agents such as extremes of temperature or fracture of the tooth give rise to impulses typically of lower voltage and slower conduction rate than those initiated by pressure on the intact tooth.

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AFFERENT IMPULSES FROM THE TEETH
RESULTING FROM A VIBRATORY STIMULUSBY CARL PFAFFMANN¹*From the Physiological Laboratory, Cambridge**(Received 5 August 1939)*

IN a previous paper [Pfaffmann, 1939] it was pointed out that tapping the intact tooth with an insulated rod produced large potential spikes in the nerve at each moment of contact and that this large potential apparently represented a synchronous volley of impulses. If, in place of these single taps, a series of mechanical stimuli are given to the tooth by means of an oscillator, a series of large potential spikes will be set up in the nerve which follows the frequency of the stimulus. The present experiments are concerned with a further analysis of this response, since it has been possible to obtain an oscillatory discharge of fairly high frequency and at the same time to analyse this response in terms of the contribution of single fibres.

APPARATUS AND PROCEDURE

The preparation and the apparatus used for the recording of nerve impulses from the teeth of the cat have already been described.

The vibratory stimulator consisted of a modified crystal loud-speaker unit activated by a heterodyne oscillator of standard make. A stylus of glass tubing mounted on the speaker movement transmitted the oscillations to the skin or tooth. Most of the unit, with the exception of the moving parts, was enclosed in a metal shield, earthed by way of the covering of the lead-in cable from the oscillator. There was no artifact picked up by the amplifier with a direct lead from this cable to earth. The stimulator was attached firmly to a heavy upright which could be moved in two dimensions by a rack and pinion adjustment.

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The excursion of the stylus for various frequencies at maximum output of the oscillator was determined directly with a microscope fitted with a micrometer eyepiece. There was a maximum excursion at 400 c./sec., but in use the response at all frequencies was equated to the displacement at 1500 cycles (0.03 mm.) by means of the volume control of the oscillator. The setting for each frequency was indicated on a scale pasted directly on the oscillator panel. These measures, however, give only an approximate idea of the stimulating conditions, since contact with the tooth produced probably some distortion.

On the other hand, it was possible to gain some idea of the accuracy of mechanical transmission from the vibrator to some more or less rigid structure. The stylus of the stimulator was placed in firm contact with the arm of a moving armature speaker unit, the leads of which were led to the pentodes of the oscillograph amplifier. The electrical fluxes generated in the coils of this unit, when the armature was moved by the stylus of the stimulator, were then registered by the oscillograph. It was found that at all frequencies up to 1500 cycles, a reasonably good sinusoidal wave was transmitted when firm contact between the two surfaces was maintained. When this connexion was loose so that a buzzing sound originated from the stylus itself, the resulting wave form became complex. Frequency halving and other changes, especially at the higher frequencies, were observed. It is believed that such distortions were adequately eliminated from the present experiments.

RESULTS

The oscillatory response recorded from the nerve can be shown to be nervous in origin by such controls as placing the electrodes on the bone of the orbital margin, crushing the nerve between the tooth and the electrodes and finally abolishing the response by novocaine. Indeed, the very character of the response shows this to be true, since the oscillatory discharge tends to have a rough character quite unlike the relatively pure sine wave produced by the stimulus itself. The fact that the electrical response decreases during a period of constant stimulation further substantiates this conclusion.

Fig. 1 illustrates the response of the whole nerve to different frequencies. For low frequencies, the composite nature of each pulse is more apparent since each cycle of the stimulus has a longer duration and gives rise to impulses for the duration of this pressure pulse. The disintegration of the response is more rapid at the higher frequencies. At any one frequency, the diminution is greater in the first few seconds

(Fig. 2). Not only is the potential magnitude reduced, but more and more gaps appear in the "frequency following" as the stimulus continues.

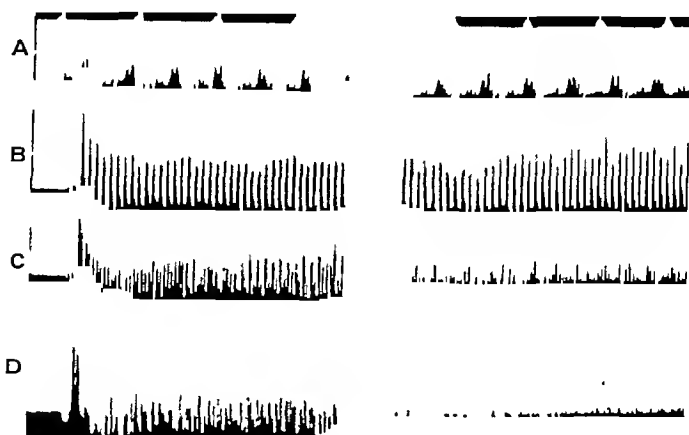


Fig. 1. The response of the whole nerve taken immediately and 10 sec. later (right-hand record). A, 80 cycles; B, 500 cycles; C, 1000 cycles; D, 1500 cycles. Time 0.02 sec.

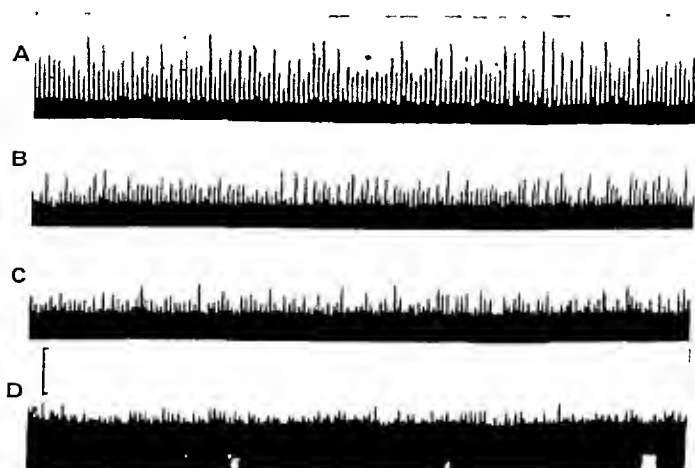


Fig. 2. Response of the whole nerve to 430 cycles. A, beginning; B, 4 sec. later; C, 8 sec. later; D, 24 sec. later. Time 0.1 sec.

The relationship between frequency and this diminution can be stated quantitatively in terms of the average potential magnitude for a constant unit of time or a constant number of cycles. Where gaps occur, these are

treated as zero values and are included in the calculation. The amplitude of the stylus excursion was maintained relatively constant as determined in the previous calibrations. Since the stylus had to be readjusted in its application to the tooth at each of the higher frequencies, in order to avoid distortions, etc., differences in the initial μV . output of the nerve recorded for different frequencies cannot have a physiological significance. In order to reduce all values to a common basis, including the response to steady pressure (which is calculated merely as the average spike height) the initial potentials in each case were treated as 100 % and the other values were adjusted accordingly (Fig. 3). Note that the greatest

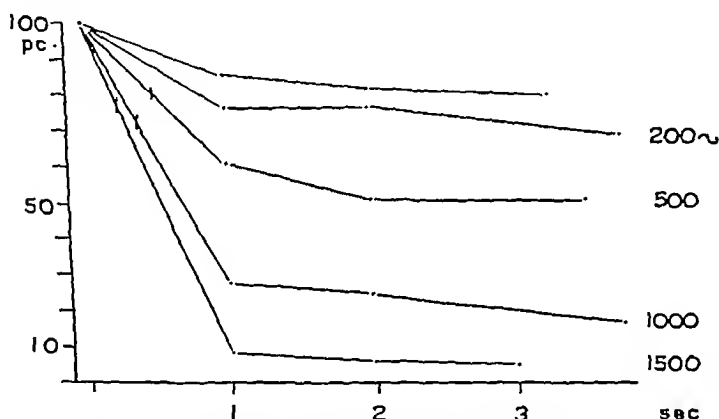


Fig. 3. The average potential magnitude at different times after the start of stimuli of different frequencies given as percentages of the initial voltage. Upper line is the response to steady pressure. Vertical lines in the three lower curves indicate where gaps occurred in the "frequency following".

diminution in response takes place during the first 1-2 sec., after which there is only a gradual fall in the curve. Similar phases in the diminution of the response of the eighth nerve upon acoustical stimulation of the cat's ear have been described by Davis [1934] and have been termed "fast equilibration" and "slow equilibration" respectively. It should be remembered that in the eighth nerve "frequency following" is accurate up to about 2500 cycles. The vertical lines drawn across the percentage curves of Fig. 3 indicate those points at which gaps in the "frequency following" begin to appear. These gaps occur when the response falls to about 75 % of its initial value. The response to 200 cycles, however, never reaches this value and never shows gaps in the "following". Such a relation is to be expected, since both aspects of the response are manifestations of the dropping out of impulses. Fewer impulses at

each cycle mean lower voltages as well as less accurate "frequency following".

In most cases, the upper limit of "following" for the response of the whole nerve was found to be in the region of 1500 cycles. Some preparations responded to this value, others just failed to, yet the writer does not place much emphasis on this exact value, since the stimulus source itself could well have been the limiting factor. It is instructive, however, to compare this value with the upper limit for the skin, which was found to be about 700 cycles under the same conditions, as shown in Fig. 4.

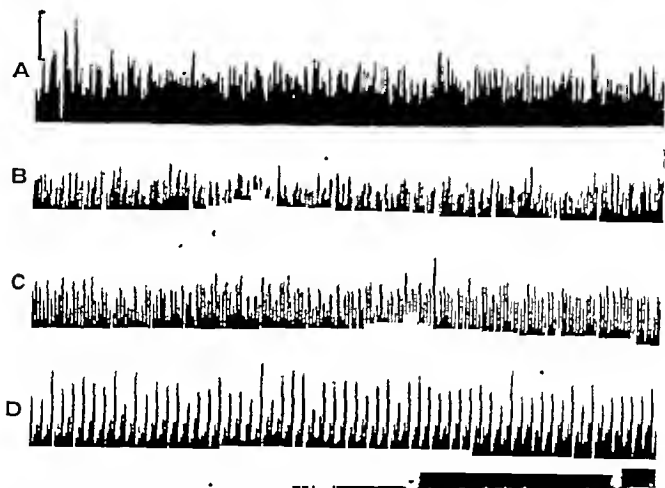


Fig. 4. Response of a cutaneous nerve to a vibratory stimulus applied to the skin. A, 800 cycles; B, 700 cycles; C, 600 cycles; D, 200 cycles. Time 0.1 sec.

This suggests that the greater rigidity of the tooth structures rather than any property of the endings themselves is responsible for the higher rate of "frequency following".

Of more interest from the theoretical point of view is the analysis of this response in terms of its unitary elements. Single fibres were obtained by cutting down the original nerve trunk. In many cases a particular strand might contain many fibres only one of which innervated a particular tooth. Restriction of the stimulus to this tooth provided a preparation which was functionally a single fibre.

It has been found, first of all, that different endings were capable of following different frequencies, and that this could be related to the other properties of the ending. Table I includes the adaptation times, as tested

TABLE I

Exp.	Adapt. time 100 g.	Max. F. pres.	Max. F. vibrat.		Tooth	Comments
			Opt.	Max.		
14 D	s	—	80	80	Canine	Only 1 or 2 widely separated to pres. Oscillator at 500 ~
17 A	0.3	180	<200	350	Canine	
20	0.3	140	0	0	Incisor	No effect with oscillator at many diff. orientations
21 D	s	300	200	360	Canine	Response only to multiples of higher frequencies
22 A	s	500	200	500	Premolar	
14 E	m	150	75	75	Canine	Response only to multiples of higher frequencies
21 B	20	300	280	400	Canine	
16 C	>300	400	400	900	Incisor	
18 B	>300	900	400	900	Premolar	
19	>300	1200	520	>900	Canine	Impulses travel in rel. ref. period of nerve

Adaptation time is given in sec., *s* means short adaptation time, usually after only a few impulses, *m* means moderate adaptation time (100 g. was not used for the stimulus).

The grouping of the adaptation times around the long and short time regions may merely be a chance selection, since endings with intermediate times were found but are not included in this table as the other relevant data were not available for them.

under standard conditions with the same stimulus (100 g.); the maximum frequency elicited by the most rapid changes of pressure that could be administered by hand ($1 \div$ minimal separation of two impulses = maximum frequency); the highest frequency obtainable with the vibrator at which there was no break-up of the "following", i.e. the optimal frequency; and finally that frequency at which only momentary "following" of the stimulus occurred, i.e. the maximal frequency. No effort was made to maintain the output of the oscillator constant in these single-fibre experiments. In general, it will be seen that those endings with the shorter adaptation times to maintained pressure had a lower maximal frequency, both to suddenly applied pressures and to the vibrating source. The limiting factor seems quite definitely to be the ending, since there is no sign, at these lower frequencies, that the impulse itself is decreasing in size as would be expected if encroachment upon the refractory period of the nerve were the cause of this disintegration of response.

It was noted that the endings with short adaptation times tended to have higher thresholds, although no accurate measurements were made.

In Exp. 20 (see table) it was impossible to obtain a response to the oscillating source in spite of the fact that many different orientations of the stimulus were tried. In Exps. 14 D, 21 D and 14 E, the response of the fibre was always some low frequency which was a simple fraction of the stimulus. Stimulation with the actual frequency corresponding with this fraction did not cause the same response because the output of the oscillator was weaker at these lower frequencies. Presumably if the energy could have been increased there would have been more accurate "frequency following". This could be demonstrated with other endings which followed the higher frequencies. If such an ending were responding to every cycle of the stimulus, reduction in the intensity led to such fractional responses as are observed in these three cases. In other words, low frequency of "following" was related to short adaptation times and high thresholds. Although there is no direct proof that these endings do not differ intrinsically in their quantitative properties, the fact that these three characteristics are related suggest that the endings are mechanically buffered to varying degrees, and that they differ in their relative accessibility to stimulation.

Fractionation is also observed in the disintegration of the response of an ending that initially follows the imposed stimulus, but cannot maintain the high rate of activity. The exact manner in which the response breaks up is determined by the frequency. As indicated in Table I, there is a given rate of stimulation for a particular ending at which there is no diminution in the response for a long period of time. With frequencies slightly higher than this there may be "following" for several seconds, after which there is a gradual fall to a frequency one-half the stimulus. With still higher frequencies there may be sudden falls to the ratio of 1 : 2 with several swings between 1 : 1 and 1 : 2 followed by a gradual break-up of the frequency. With still higher frequencies, more rapid disintegrations may occur (Fig. 5). At the highest frequencies, there will be no response at all in the 1 : 1 range. Fig. 6 illustrates graphically the response of another single-fibre preparation to varying frequencies. In this case it must be admitted that there are two factors involved in the reduction of the nerve response, namely, frequency and intensity of the stimulus. Since the output of the oscillator decreased for the higher frequencies, the recorded disintegration of the response at these higher frequencies takes place more rapidly than would occur if the intensity were constant. On the other hand, in another experiment (no. 17) the maximum frequency at which any "following" occurred was 350 cycles. This is below the maximal output of the stimulator, and therefore

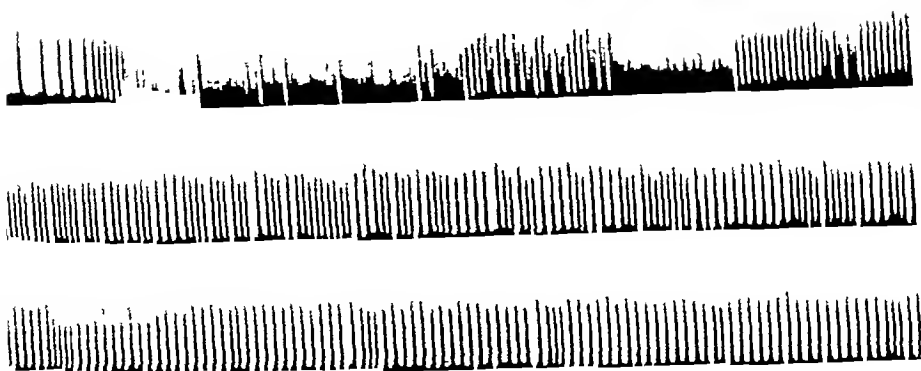


Fig. 5. Response of a single fibre. A. Optimal frequency, 550 cycles. B. Beginning of response to 880 cycles. Stimulus started by quickly turning up the volume control of the oscillator from zero to maximum which accounts for the gradual development of the high-frequency response. C and D. Continuous with B. Note the 1:1, 1:2, and finally the 1:3 response. Time 0.1 sec.

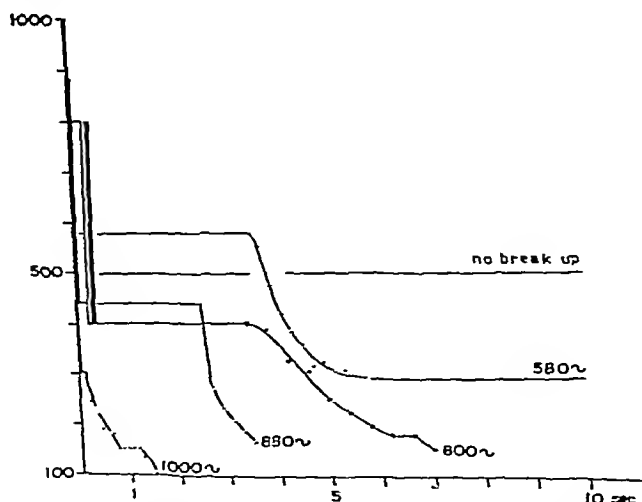


Fig. 6. Single fibre response to different frequencies. The dark vertical lines of the graph represent sudden shifts from 1:1 to 1:2 response. Note that the 1000 cycle response dropped immediately to 1:5. 500 cycles is the optimal frequency. The responses were not followed beyond the times indicated on the graphs except the 1000 cycle response which ceased at 1.5 sec.

reduction in the intensity of stimulation as frequency was increased could not be the limiting factor. For frequencies below 350 cycles essentially the same behaviour as that described above was observed. In these cases the increase in intensity for the higher frequencies would tend to delay the disintegration.

Fig. 5 is of additional interest because it indicates that the nerve ending may be driven to respond at a rate which causes the nerve to conduct in its relative refractory period. It will be observed that, for any one train of impulses at 1 : 1, there is a progressive reduction in the size of the impulse height, which recovers after the omission of one impulse. This is a good demonstration of the progressive falling off in the potential height in a train of impulses at high frequency in a single fibre similar to that observed in the whole nerve [Gasser & Grundfest, 1936]. Even when conducting at the ratio of 1 : 2, the impulse is still travelling in the relative refractory period as indicated by the reduction of spike height. From this it can be calculated that the relative refractory period of this fibre must extend over 1.1–2.3 msec. Similar values are obtained from other preparations. Fibres in class A have been shown to have an absolute refractory period of 0.5 msec. with a complete recovery after 3 msec. [Gasser & Grundfest, 1936]. C fibres, on the other hand, have absolute refractory periods of 1.8–2.0 msec. [Grundfest & Gasser, 1938]. Thus these fibres belong to the fast conducting group, which agrees with the estimated velocities of the impulses produced by pressure on the intact tooth [Pfaffmann, 1939].

DISCUSSION

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total potential amplitude of response to varying frequencies with constant amplitude stimulus, recorded on films run at slow camera speed. Fig. 7 shows the relation between the degree of equilibration and frequency. Note that the curves show a progressive fall compared with a rise in the similar curve based on the eighth nerve response as found by Derbyshire & Davis [1935].

It must be pointed out that certain precautions should be observed when treating the results in such a manner. In some cases there was an apparent increase in the potential magnitude rather than the decrease

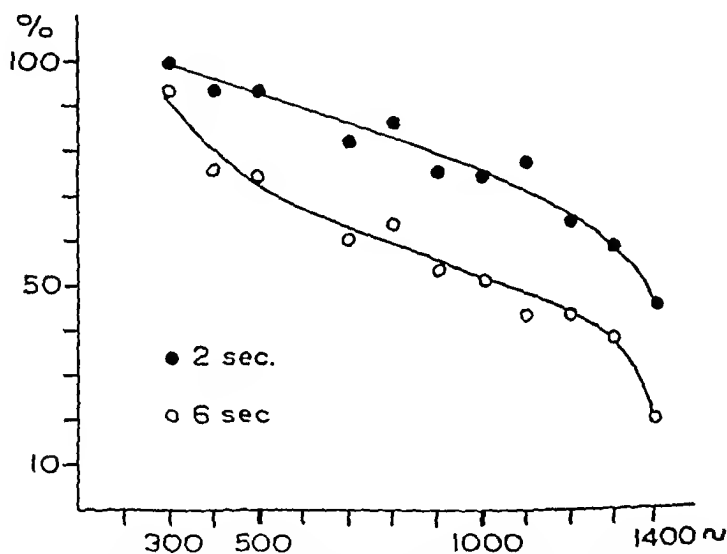


Fig. 7. The percentage of initial response remaining after 2 sec. and after 6 sec. stimulation at different frequencies. These curves indicate the degree of fast equilibration and slow equilibration respectively.

related to equilibration. Closer examination shows that this is caused by a coincidence of the alternating phases of the nerve response with the result that there is an almost complete lack of potential in the alternate cycles of the response. A similar coincidence of the phases of alternation were observed by Pumphrey & Rawdon Smith [1936] and Echlin & Fessard [1938].

With these qualifications and considerations in mind, it seems clear that the evidence of these experiments provide additional support for the volley theory of auditory nerve function.

One finding that was not altogether expected was that endings with longer adaptation times responded more accurately to the higher fre-

quencies of stimulation. It would appear that the postulated rapid adaptation of the eighth nerve terminations [Adrian, 1932; Davis, Derbyshire, Lurie & Saul, 1934] is not a necessary condition for the "frequency following" manifested by that nerve. The fact that the non-auditory endings of the inner ear [Ross, 1936] as well as the phylogenetically related lateral line endings [Hoagland, 1935; Sand, 1937] are slow or even non-adapting in type is interesting in this connexion.

Throughout the preceding discussion we have concerned ourselves solely with the physiological basis of this response to an oscillating stimulus, without regard to the relation this may have to the general behaviour of the animal. It is reasonable to suppose that this oscillatory discharge is related to the peripheral basis for the perception of vibration, although it is not the present intention to consider this aspect of the problem [see Echlin & Fessard, also Newman *et al.*]. When the stimulator used in these experiments was applied to the experimenter's own tooth there was a complex sensation resulting from tactile plus auditory stimulation. The direct tactile perception of vibration disappeared before the auditory one as the frequency was increased. Because the present apparatus emitted a definite note at the high frequencies, there could be no clear separation of these two effects. At no time was the experience painful, although it has been shown that vibratory stimulation applied to the skin can give rise to pain at the extreme intensities [Knudsen, 1928].

SUMMARY AND CONCLUSIONS

1. Application of a vibrating stylus to the surface of the intact tooth gives rise to an oscillatory discharge in the dental nerves which is synchronized with the frequency of the stimulus.

2. With a maintained stimulus, the amplitude of this response falls off together with a decrease in the accuracy of "frequency following". The major part of this diminution or "equilibration" takes place during the first few seconds of activity and the effect becomes progressively greater with the higher frequencies.

3. The upper limit of "frequency following" for the whole nerve in these experiments has been about 1500 cycles. This exact value is probably determined by the characteristics of the stimulating mechanism itself. Since soft tissues of the skin yield maximal values of 700 cycles under the same conditions, it is concluded that the higher values for the tooth result from the greater rigidity of the structures concerned.

4. Different endings, as indicated by single fibre responses, have different maximal frequencies of response to the oscillating source (from

80 to 900 cycles) which can be related to other properties of the ending, notably the maximum frequency of response to rapid pressure changes and the adaptation time to a standard pressure.

5. Single fibres when stimulated at rates higher than their optimal frequency show various types of fractional response, 1:2, 1:3, etc., which develop gradually at frequencies just supra-optimal and more rapidly and suddenly at still higher frequencies.

6. In some cases, the endings may be activated at frequencies overlapping the relative refractory period of the nerve.

7. The relation of these results to other oscillatory nerve discharges is discussed, especially with respect to that of the auditory nerve.

The writer wishes to thank Prof. Adrian for his constant help and encouragement and Dr Matthews for his ever-willing advice.

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THE DIFFUSION OF CHLORIDE AND GLUCOSE INTO FROG MUSCLE

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IN a recent review Fenn [1936] concluded that there was good evidence for the belief that *in vivo* chloride is confined to the extracellular water of muscle. Since that time further evidence has been adduced in support of this conclusion by M. G. Eggleton [1937] for the cat, by Hastings & Eichelberger [1937] for the dog, and by Manery & Hastings [1939] for the rat and rabbit. Gersh [1938] has also supported this view on anatomical grounds. The general conclusion may be stated approximately to be that in the muscles of frogs and laboratory mammals chloride in the plasma is in diffusion equilibrium with the extracellular phase of muscle, constituting 10-20 % by weight of the tissue.

In investigations of the diffusion of chloride into isolated frog muscle Eggleton, Eggleton & Hamilton [1937] were able to show conclusively that the chloride in the muscle was in true diffusion equilibrium with the chloride in the Ringer solution bathing the muscle. But they obtained figures for the "chloride space" in the muscle which were constantly in excess of those obtained *in vivo*, e.g. 29-36 % of the muscle (confirming Fenn, Cobb & Marsh's [1934] figures of 25-37 %), whereas the figures obtained *in vivo* are in the neighbourhood of 10-15 % [Fenn *et al.* 1934]. These latter figures are in reasonable agreement with the histological estimates of the extracellular phase [Fenn, 1936; Gersh, 1938]. As the increase in "chloride space" found *in vitro* occurs in isotonic Ringer solution and is not accompanied by any significant change in the weight of the muscle it has proved very difficult to interpret.

In the course of another investigation we have made some observations on the "chloride space" and the "glucose space" of the same frog gastrocnemius which throw a little additional light on this problem.

METHODS

The muscle has been soaked in 50 c.c. Ringer solution containing 0.53 % NaCl, 0.012 % KCl, 0.021 % CaCl_2 , $6\text{H}_2\text{O}$, 0.02 % NaHCO_3 and 1 % glucose. The solution has been maintained at 2–3° C. during the soaking and the muscle has been carefully dried on no. 50 Whatman filter paper and weighed before and after soaking. The muscle has been extracted by grinding with washed sand under 10 % trichloroacetic acid, and the extract made up to contain a final concentration of 2 % trichloroacetic acid. Each muscle was extracted four times.

Chloride has been estimated by the titrimetric iodometric method of Sendroy [1937], controls having shown that neither sand nor trichloroacetic acid yielded any interfering substances. Reducing substances have been determined by the Hagedorn-Jensen method on neutralized aliquots of the trichloroacetic acid filtrate. Here again, neither sand nor trichloroacetic acid produces any interference. Non-fermentable reducing substances have been determined by the method described by Van Slyke & Peters [1932]. Controls have shown that sodium trichloroacetate does not interfere with the removal of glucose from aqueous solution by washed yeast cells.

RESULTS

When the isolated gastrocnemii of Hungarian frogs were soaked in isotonic Ringer solution containing 1 % of glucose the "glucose space", even when calculated on the assumption that all the reducing matter in the muscle extract was glucose, was consistently less than the "chloride space". That this phenomenon was not due to glycolysis was shown by soaking a pair of muscles in Ringer solution for the same time, grinding one as rapidly as possible under ice-cold trichloroacetic acid in an ice-cold mortar, and grinding the other in a leisurely manner under trichloroacetic acid at room temperature. The precautions to minimize glycolysis had no effect on the observed "glucose space".

Taking the fermentable reducing matter to be glucose, the results obtained in a series of experiments in which muscles were soaked for varying lengths of time are summarized in Table I. The figures in this table represent values of the quantity:

$$100 \times \frac{\text{mg./g. muscle}}{\text{mg./c.c. Ringer}}$$

There are several points of interest in this table. In the first place, although Eggleton *et al.* [1937] showed that chloride reached equilibrium with the muscle after 30 min. soaking at 2–3° C., our figures show a rise

TABLE I

(1) Time of soaking hr.	(2) Chloride permeation	(3) Glucose permeation	(2)-(3)
25	41	34	7
25	33	27	6
23	40	32	8
23	46	33	13
23	40	36	4
23	35	34	1
			Mean 6
8*	35	27	8
8	34	29	5
8	36	27	9
8	37	27	10
			Mean 8
4	29	24	5
2	29	21	8
			Mean 6.5

The mean values in the last column do not differ significantly from one another.

* Ringer saturated with O_2 .

in chloride permeation with time. At 2-4 hr. our figures are the same as earlier figures. Subsequently the "chloride space" increases, apparently in two distinct steps, i.e. it rises from 29 to 33-37 %, and then in some instances to 40-46 %. All our muscles were irritable when removed from the Ringer solution and no significant change in weight occurred during soaking. Further, in the instance marked with an asterisk in Table I the Ringer solution was saturated with O_2 and in contact with an O_2 atmosphere during the immersion of the muscle. This oxygenation experiment lends no support to the view that the increase in chloride permeation is due to progressively developing anaerobiosis in the muscle. It appears more likely that it is due to the dilution of some factor responsible for the common impermeability to chloride of a series of barriers, membranes or interfaces between different phases of the muscle substance.

The concept of a series of barriers in the muscle seems to be supported by the data for glucose permeation. Here it is seen that the observed permeations fall into three groups, 21-24, 27-29, and 32-36 %, the latter two groups being identical with the two earlier groups of chloride permeations.

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			<hr/> Mean 8
4	29	24	5
2	29	21	8
			<hr/> Mean 6.5

The mean values in the last column do not differ significantly from one another.

* Ringer saturated with O_2 .

in chloride permeation with time. At 2-4 hr. our figures are the same as earlier figures. Subsequently the "chloride space" increases, apparently in two distinct steps, i.e. it rises from 29 to 33-37 %, and then in some instances to 40-46 %. All our muscles were irritable when removed from the Ringer solution and no significant change in weight occurred during soaking. Further, in the instance marked with an asterisk in Table I the Ringer solution was saturated with O_2 and in contact with an O_2 atmosphere during the immersion of the muscle. This oxygenation experiment lends no support to the view that the increase in chloride permeation is due to progressively developing anaerobiosis in the muscle. It appears more likely that it is due to the dilution of some factor responsible for the common impermeability to chloride of a series of barriers, membranes or interfaces between different phases of the muscle substance.

The concept of a series of barriers in the muscle seems to be supported by the data for glucose permeation. Here it is seen that the observed permeations fall into three groups, 21-24, 27-29, and 32-36 %, the latter two groups being identical with the two earlier groups of chloride permeations.

We were able to show in one experiment in which a pair of gastrocnemii were immersed in small volumes of Ringer solution that the uptake of glucose by the muscle is very rapid during the first 10 min., and thereafter

occurs at a rate too small to be measured over a period of several hours. The results of this experiment are given in Table II, in which the figures represent the percentage of the muscle with which the amount of glucose disappearing would come into equilibrium.

TABLE II

Soaking time min.	Equivalent permeations	
	Muscle A	Muscle B
10	21	23
120	21	23
240	21	23
380	24	29
480	28	29

Comparison of these figures with those of the previous table supports strongly the presumption that the permeations obtained in 2-4 hr. represent an equilibrium state, and that the subsequent changes are due to increase in the permeability of the muscle occurring in stages. This conclusion is further supported by consideration of the difference between the glucose and chloride permeations. In every instance the chloride permeation is the higher, but, as shown in the fourth column of Table I, there is no regular tendency towards a change in the mean difference with increasing time. A difference in the same sense would be expected were the more rapid diffusion of chloride into muscle an important factor during the time period under consideration. However, were this so, the difference between the two permeations should diminish with increasing time. The absence of such a diminution, together with demonstration of the rapid diffusion of glucose into muscle, appears to dispose of this hypothesis. The alternative appears to be that the successive increases in permeation are due to successive breakdowns of barriers to diffusion within the muscle, followed by rapid diffusion across the site of the barrier. It would also appear legitimate to suggest that each barrier becomes ineffective against chloride at a time when it can still prevent the diffusion of glucose. The mean values for the stages of permeation found for glucose and chloride are summarized below:

Stage of permeation ...	I	II	III	IV
For glucose	22 (4)	23 (7)	34 (5)	—
For chloride	—	29 (2)	35 (6)	43 (4)

(The figures in parentheses refer to numbers of observations)

One other point arises from these data. The minimum fraction of the muscle water with which a diffusible substance in the external medium

can come into equilibrium is the extra-cellular fraction. Since *in vitro* chloride constantly permeates more of the muscle than does glucose, it follows, without reference to any histological criterion, that *in vitro* chloride must diffuse into some part of the muscle cells. It becomes a matter of some interest to know what factor normally operative in the organism to maintain the chloride impermeability of the muscle cells is diluted or destroyed during soaking in Ringer solution.

SUMMARY

The diffusion of chloride and glucose into the gastrocnemii of Hungarian frogs occurs *in vitro* in stages which are similar in magnitude for the two substances but separated in time. The chloride permeation of the muscle is always greater than the glucose permeation. The implications of these findings are discussed.

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FATTY INFILTRATION OF THE LIVER IN PREGNANT EWES

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THROUGHOUT the world sheep breeders suffer losses among their breeding ewes from a disease variously known as "pregnancy disease", "pregnancy toxemia", and "twin lamb disease". The two most characteristic clinical features of this disease are a marked ketosis and extreme fatty infiltration of the liver. In previous papers [Fraser, Godden, Snook & Thomson, 1938, 1939] it was shown that ketosis can be produced in pregnant ewes by undernutrition, and the possible relation between the experimental condition and pregnancy disease was discussed. The ketosis was associated with marked fatty infiltration of the liver and opportunities were taken to make quantitative and qualitative studies of the fat present in the livers of normal and abnormal experimental animals, and also in livers from field cases of pregnancy disease.

It is sometimes stated that a certain degree of fattiness of the liver is a normal physiological associate of pregnancy. Thus Gaiger & Davies [1932] list among the various causes of fatty infiltration—"Physiological—during pregnancy and lactation". Little definite evidence on this point is available, however. The work of Coope & Mottram [1914] has been quoted as showing that fatty infiltration of the liver is a normal concomitant of pregnancy but the increases shown (in cats and rabbits) are relatively small and, in view of recent work, of doubtful significance. Thus they found 3.4 and 5.6 % total fatty acid in the livers of two pregnant rabbits and an average of 2.6 % in controls (7) of mixed sexes. Increases of this order do not warrant use of the term "fatty infiltration". Best & Ridout [1933] mention the assumption that pregnant rabbits near term tend to have fatty livers, but note that a colleague was unable to confirm this, even when the does were kept on a diet high in fat.

No work has been published concerning the chemical nature of the excess fat found in the livers of ewes afflicted with pregnancy disease. Indeed, for reasons mentioned above, it has been suggested that fattiness of the liver might be characteristic of healthy pregnant ewes near term. In the current investigation the opportunity was taken to estimate the amount and nature of the fat present in the livers of normal sheep (wethers, barren ewes, healthy pregnant ewes), apparently healthy but ketonaemic ewes, comatose, ketonaemic ewes, and field cases of pregnancy disease. In all cases concurrent analyses were made on the blood, and for the experimental animals data were available concerning food consumption, bodyweight changes and the composition of the blood over a considerable period prior to slaughter. This supplementary information is of prime importance in interpreting results. Details concerning the feeding and management of the experimental ewes have been reported elsewhere [Fraser *et al.* 1938].

METHODS

The liver was removed from the animal as soon as possible after slaughter or death. Small cubes of tissue for subsequent histological examination were cut from the various lobes and placed in 10 % formaldehyde. The fresh liver was then minced and thoroughly mixed.

DETERMINATIONS

Moisture. Representative samples of the fresh pulped liver were dried to constant weight at 100° C. in an electric oven.

Fatty substances. 10–14 g. samples of the fresh pulped liver were accurately weighed and then ground in a mortar with anhydrous sodium sulphate. The dry powder so obtained was extracted with chloroform for 4 hr. in a Soxhlet apparatus, the material was then re-ground and extracted for a further 4 hr. The filtered chloroform extract was then made up to 100 c.c. in a measuring flask, aliquots being used, as needed, for the following estimations:

(a) *Total fat.* The chloroform was removed by distillation and the residue dried to constant weight at 100° C.

(b) *Total phosphorus.* Five c.c. of the chloroform extract were placed in a small Kjeldahl flask, the chloroform was removed by gentle heating and the residue was then digested with 5 c.c. of a mixture of equal parts of concentrated sulphuric and nitric acids until the liquid was colourless and white fumes were given off. On cooling, water was added to the

residue, which was then neutralized, and in it phosphorus was estimated by the method of Fiske & Subbarow [1925].

(c) *Unsaponifiable residue.* Suitable aliquots were taken and the chloroform removed. 25 c.c. of *N*/2 alcoholic potash were then added to the residue and the mixture boiled until saponification was complete. Water was added and the unsaponifiable residue removed by 6 extractions with ether. The combined ethereal extracts were washed with water until neutral, the ether was removed and the unsaponifiable residue determined by weighing to constant weight after drying *in vacuo* at 100° C.

(d) *Iodine number.* Dam's pyridine-sulphate-bromine method was used [Plimmer, 1938].

(e) *Glycogen.* The glycogen content of the fresh liver was not determined, but as soon as possible after slaughter of the animal small samples of liver tissue were placed in absolute alcohol and preserved until it was convenient to complete the analyses. The liver slices were then drained on blotting paper, and weighed by difference in tubes containing 60 % KOH. Subsequent procedure was similar to that described by Evans, Tsai & Young [1931] but 80 % alcohol was used for precipitation, the sugar in the ultimate hydrolysate being estimated by the Somogyi modification of the Shaffer-Hartmann method [Peters & Van Slyke, 1931].

RESULTS

To serve as a guide to the possible effect of pregnancy on the fat content of the liver, a number of small laboratory animals on stock diets were slaughtered just before parturition, the livers being removed and analysed. None of the livers from these small, healthy animals contained unusual amounts of fat, the maximum being 21.6 % total extract in the dry matter of one of the rat livers. The average values are given in Table I. From these data it does not appear that there is any fatty infiltration of the liver near term in the rat, rabbit or guinea-pig.

TABLE I. The fatty substances in the livers of pregnant rats, rabbits and guinea-pigs

	Number of foetuses	Moisture %	Total fat	
			Wet liver %	Dry liver %
Average: 8 rats	9	72	5.0	17.6
3 rabbits	7	72	4.7	16.7
Guinea-pig A	3	72	2.8	10.0
B	3	71	2.6	9.0

(For comparison. Best, Channon & Ridout [1934] found 5.4 % total extract in the fresh livers of grain-fed rats used as controls.)

Samples of liver were obtained from healthy wether hoggets (castrated male sheep) being slaughtered for human consumption. These were

analysed to give an index of the lipids present in the livers of healthy sheep undisturbed by any sexual activities. It can be seen from Table II that the livers of healthy wether hoggets contain essentially the same percentage of moisture and total chloroform extract as those of pregnant rats and rabbits. No fat could be detected by histological examination following staining of the tissue with Scharlach R.

TABLE II. The moisture, fat and glycogen content of sheep livers

	Moisture %	Total fat		Glycogen in liver preserved in alcohol %
		Wet liver %	Dry matter %	
Average: 2 healthy wethers	73	4.8	18	—
Healthy barren ewe; basal diet (No. 55, Group III, 1939)	70	5.6	19	6.0
Healthy fat pregnant ewe killed near term (No. 30, Group I, 1939), 3 lambs	74	5.8	22	3.3
Average: 11 non-ketonaemic ewes at term (1-3 lambs)	75	5.5 (range 5-7)	22	—
"Healthy" ketonaemic ewe (No. 23, Group IV, 1939), 3 lambs	64	16.6	43	1.2
Ketonaemic ewe killed im- mediately after lambing (No. 4, Group V, 1938), 2 lambs	57	31	71	—
Average: 5 ketonaemic ewes killed when comatose (2-3 lambs)	60	25 (range 20-32)	62	Negligible
Average: 5 field cases pregnancy disease (2-3 lambs)	66	17 (range 15-20)	51	—

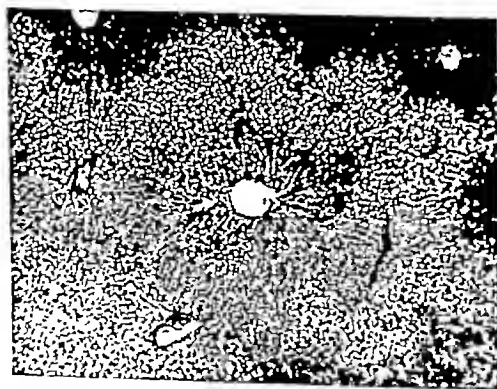
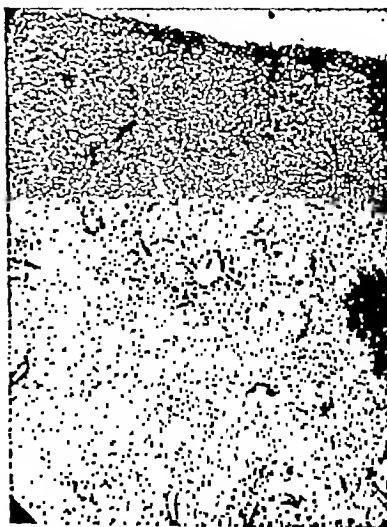
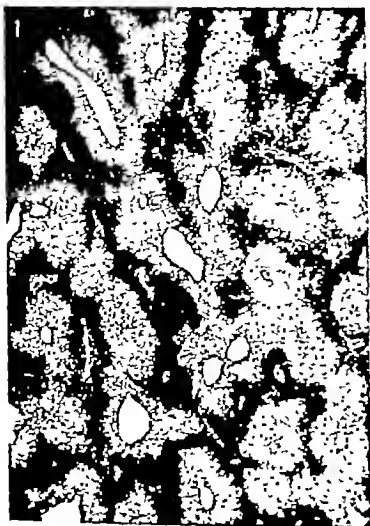
An experimental barren ewe (No. 55, Group III, 1939) was slaughtered for carcass examination after being for 3 months on a basal ration which produced ketosis in pregnant ewes [see Fraser *et al.* 1939]; the animal had also been fasted for 2 days, a week prior to slaughter. It might have been expected that the fasting and continued low plane of nutrition would have produced fattiness of the liver but it can be seen from Table II that the analytical figures are comparable with those of the wethers. Here again histological examination indicated that the slight fat content was distributed uniformly throughout all the cells.

A fat, experimental, pregnant ewe was slaughtered for carcass examination 1 day before the due date of lambing. This animal (No. 30, Group I, 1939) had been well fed throughout gestation and had gained 37 lb. in bodyweight. She appeared to be in the best of health up to the time of slaughter and at autopsy all organs appeared normal. Three

healthy lambs (total weight 19 lb.) were carried. As can be seen from Table II there was no significant increase in the fat content of the liver. It was interesting to find on histological examination, however, that there was a suggestion of accumulation of fat in the cells adjacent to the portal vessels. As will be described later, it has been found that fatty infiltration of the liver in the ewe appears to progress from the periphery of the lobule towards the centre, and there was therefore some indication here of incipient fatty infiltration. This finding links up with the changes noted in the composition of the blood. Four days prior to slaughter blood drawn from this ewe contained 48 mg. sugar/100 c.c. and no ketone bodies were detected. On the day of slaughter, however, the blood sugar had dropped to 37 mg. and 9 mg. total ketone/100 c.c. blood were present. These blood figures are of interest in connexion with the glycogen content of the liver. It will be noted that the liver of the barren ewe contained 6.0 % glycogen in contrast to the 3.3 % found in the liver of the pregnant ewe receiving a much better ration (daily intakes, 0.65 and 1.69 lb. starch equivalent respectively). When stained with Best's carmine, sections from the liver of the barren ewe appeared deep red in colour, all cells containing glycogen. In contrast, the staining showed that in the liver of the multiply-pregnant ewe the glycogen was concentrated in the cells around the portal vessels. It seems that the strain of multiple pregnancy tends to exhaust the glycogen reserves of the liver and this may predispose to fatty infiltration near term. However, this ewe was only 1 day antepartum when killed and the analytical data do not indicate fattiness of the liver.

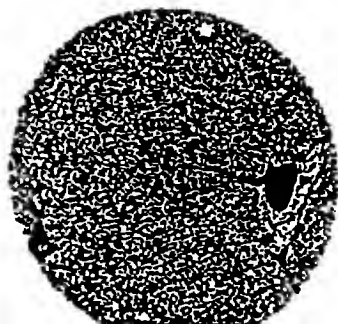
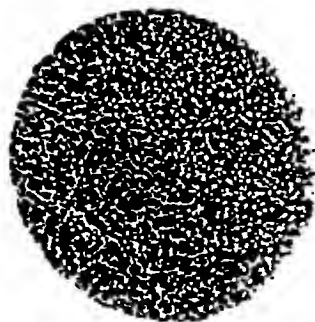
During the main investigation, it was possible to obtain liver samples from a number of non-ketonaemic ewes which, for various reasons, had to be slaughtered at, or soon after, parturition. Some of the experimental animals, for example, were excessively fat and had trouble at lambing, being slaughtered for such reasons as excessive haemorrhage or a torn uterus. These ewes cannot be classed as strictly normal, but blood analyses had shown that they were not ketonaemic and it therefore seems that one is justified in considering them as controls for comparison with ketonaemic animals. It will be seen from the table that none of these animals had fatty livers and the data lend support to the suggestion that fatty infiltration of the liver is not a normal concomitant of pregnancy. Histological examination showed that the fat in the livers of non-ketonaemic pregnant ewes was evenly distributed throughout the cells.

Ketosis results both when ewes are kept on a quantitatively inadequate ration throughout gestation and when overfat, pregnant ewes are



4

5



suddenly placed on reduced rations. This ketosis, which may become very pronounced, is preceded, or accompanied, by fatty infiltration of the liver. Thus an experimental ewe (No. 23, Group III, 1939) receiving a quantitatively inadequate diet was slaughtered 14 days prior to due date of parturition. This animal had been markedly hypoglycaemic and ketonaemic for about a month, and on the day of death the blood contained 48 mg. total ketone/100 c.c. As can be seen from the table the liver contained excessive fat (16.6 % in the wet liver) and only 1.2 % glycogen. Microphotograph No. 1 shows the distribution of the fat in the liver of this apparently healthy ewe. It will be seen that the fat is accumulated in the cells around the periphery of the lobules, the tissue around the central vein being normal. This distribution is characteristic of that seen in the livers of all ketonaemic ewes, both experimental and field cases.

Many multiply-pregnant ketonaemic, experimental ewes went to term, and although the lambs were very poor, the ewes did not necessarily display any untoward symptoms. This is illustrated by ewe No. 4. A fortnight prior to lambing the blood from this animal contained 24 mg. sugar and 64 mg. total ketone/100 c.c.; the day before lambing the ketone bodies had increased to 83 mg./100 c.c. An uneventful lambing took place and immediately afterwards the beast was slaughtered. The carcass was emaciated but the liver was the only abnormal organ; this was very pale and extremely fatty (71 % total extract in the dry matter). Some idea of the degree of infiltration may be obtained from microphotograph No. 2 which shows that only a few cells around the central vein were normal. As the process of infiltration had probably been proceeding over a period of many weeks, it would appear that this accumulation of fat does not have any serious effect on liver function.

A number of ketonaemic experimental ewes lost their appetite and with decreased food intake serious clinical symptoms became apparent. Anorexia was followed by hyper-ketonaemia, lethargy, partial blindness, twitching of the ears, grinding of the teeth, physical weakness and coma. The affected animals remained comatose for several days and most of them were slaughtered when apparently dying. Several actually lambed but did not recover postpartum and died within 2 days. Autopsy revealed emaciated carcasses and marked fatty infiltration of the liver. In Table II are given the average figures for 5 such ewes which were slaughtered antepartum when comatose. In microphotograph No. 3 a section is shown which was prepared from a liver containing 68 % fat in the dry matter. A better index of the degree of the fatty infiltration in

the cells of this liver is obtained from the high-power microphotograph No. 4, from which it can be seen that in many of the cells the protoplasm appears to have been almost entirely replaced by fat, the nucleus being pushed right up against the cell wall. This last illustration should be compared with microphotograph No. 5, which shows a section prepared from the liver of a healthy, non-ketonaemic ewe slaughtered at lambing because of a torn uterus.

TABLE III. Weight of total fatty substances present in livers of normal and ketonaemic ewes

No.		Weight of liver lb.	Total fat %	Weight of total fat in the liver lb.	Number of lambs
Normal ewes					
55.	Barren	1.5	5.6	0.08	—
30.	Healthy, fat, pregnant	2.2	5.8	0.13	3
13.	" " "	2.4	5.6	0.13	1
77.	" " "	2.5	7.2	0.18	3
Ketonaemic ewes					
23.	Killed for carcass	1.9	16.6	0.32	3
35.	Killed when dying	2.6	24.0	0.62	3
92.	" " "	2.8	26.7	0.75	2
328.	" " "	2.6	25.3	0.66	2
58.	Died after lambing	2.9	21.1	0.61	2
52.	Died after prolonged anorexia	3.2	32.1	1.02	3

Not only does the percentage of fat increase in the liver of an ewe which becomes ketonaemic but the liver tends to be enlarged. A good index of the amount of fat which accumulates in the liver is obtained from Table III. Here the total weight of fat present in the various livers is given.

Blood and liver samples were obtained, whenever possible, from field cases of pregnancy disease. The animals were always markedly ketonaemic, and chemical and histological examination of the fatty livers indicated that the changes produced in the field were similar to those found in experimentally-produced ketosis. Complete loss of appetite was also a characteristic feature of those field cases available for treatment. It is interesting to note that the hypoglycaemia and ketonaemia in field cases of pregnancy disease could be corrected by glucose therapy (oral or intravenous), but the animals would not resume eating and eventually died. The livers were still markedly fatty, even where ketonaemia had been eliminated for several days by glucose treatment.

POSSIBLE NECROTIC CHANGES

In all cases (experimental and field) where ewes died after displaying symptoms characteristic of pregnancy disease, a careful search for possible necrotic changes in the liver was made, using paraffin sections cut at 7μ . Prof. J. S. Young of the Department of Pathology, Aberdeen University, kindly examined a number of representative sections and reported that in no case did it appear that the fatty infiltration was accompanied by degenerative changes in the cell structure. This confirms the work of M'Fadyean [1924] who, as the result of histological examination, stated that the liver changes seen in pregnancy disease in ewes, resulted from extreme physiological infiltration and not a toxic degeneration.

CHEMICAL CONSTITUTION OF THE LIVER LIPIDS

Various chemical analyses were carried out to test the hypothesis that the fat in the livers of ketonaemic ewes had been transported thereto from the body depots. Phospholipins are considered to play a role in the transport of fat in the body and phosphorus determinations were therefore made on the chloroform extracts in view of the possibility that the fatty infiltration may have been due to an upset in phospholipin metabolism. The results are given in Table IV.

TABLE IV. The phosphorus and unsaponifiable extract present in the chloroform extract of sheep livers

	Total fat in dry matter %	P as a percentage of		Unsaponifiable residue in total fat %
		Total fat %	Non-fat dry matter %	
Normal livers				
Healthy wether	17	2.1	0.43	8.5
" "	18	1.9	0.43	8.5
Ewe 93	21	—	—	7.8
85	25	2.0	0.66	8.6
Fatty livers				
Ewe 35	62	0.42	0.68	3.9
7	56	0.47	0.60	4.3
3	68	0.30	0.65	—
4	71	0.32	0.71	3.8
81	61	0.44	0.69	4.0

It was not considered likely that the infiltration in the ewe was due to unbalanced sterol metabolism but, to obtain definite evidence on this point, the amount of unsaponifiable residue in various chloroform extracts was determined. The results are included in Table IV.

It will be seen that the extract from the fatty livers contains much less phosphorus per unit than the extract from normal livers. This is

what one would expect if the excess fat was normal body fat transported from the depots. A better guide to abnormality is obtained by expressing the phosphorus as a fraction of the non-fatty dry matter of the liver. On this basis of comparison, the phospholipins in the fat-infiltrated livers are of the same order as that found in the liver of the normal pregnant ewe No. 85.

Best, Channon & Ridout [1934] found 8 % unsaponifiable extract in the liver fats of normal "control" rats, which corresponds to that found in the liver fat of normal sheep. As one would expect following the infiltration of depot fat, the percentage of unsaponifiable residue in the liver fats decreases as the fat content of the liver increases. It is apparent that the fatty liver produced in the experimental ewes, or found in cases of pregnancy disease, is not of the cholesterol ester type.

As a further test regarding the nature of the fat present in the fatty livers, Iodine Numbers of the extracts were determined. No definite correlation was apparent between the I.N. of the extract and the percentage of fat in the liver, but there was an indication that with increased fatty infiltration the I.N. tends to fall until it approximates that of depot fat. Thus the extracts from 5 fatty livers had I.N.s ranging from 77 to 71 as compared with 93 for the extract from the liver of the healthy ewe (No. 30, Group I, 1939) carrying 3 lambs.

DISCUSSION

It seems that the ketosis produced in pregnant ewes by undernutrition follows, or is associated with, the mobilization of depot fat and its accumulation in the liver. Such data as are available also indicate that this fatty infiltration is associated with depletion of the liver glycogen. The finding that the liver of the poorly-fed barren ewe contained about double (6 %) the glycogen found in that of the well-fed, pregnant ewe slaughtered 1 day antepartum supports the hypothesis that in the terminal stages of multiple pregnancy, foetal demands prevent the storage of the usual amounts of glycogen. Naturally, if the glycogen reserves are depleted, the animal is less able to withstand undernutrition, and if the food supply is restricted, a need to use depot fat will quickly arise. Thus the liver of the ketonaemic ewe (to all appearances healthy) which was slaughtered for carcass examination contained only 1.2 % glycogen but about twice the normal amount of fat. With continued undernutrition the ketonaemia becomes increasingly severe, and it can be assumed that this is associated with even greater depletion of the glycogen reserves and increased accumulation of fat in the liver.

There is reason to doubt whether loss of liver function can be of any significance in precipitating the decline of ketonaemic animals, as various workers have shown that the vital functions can be carried out by only a small fraction of the liver. Thus Bollmann & Mann [1935] excised up to 80 % of the liver from experimental dogs and found that normal functions could be carried out even when regeneration was prevented. Similar retention of function has been seen in animals with cirrhosis sufficient to destroy a large portion of the liver. Bollmann & Mann have shown, however, that it is the detoxicating power of the liver which is most seriously affected when the liver becomes infiltrated with fat, also that livers lacking in glycogen are less able to cope with toxic agents. Bollmann [1938] discusses further work which suggests that the detoxicating power of the liver may be lost before other functions fail. It is therefore possible that lack of liver glycogen may be a factor in reducing ketonaemic ewes to a comatose condition, either directly because of the resultant weakness, or indirectly by a loss of detoxicating ability by the liver cells. There is fairly definite evidence that fatty infiltration in itself will not result in any harmful symptoms. Many severely ketonaemic ewes, which by inference had fatty livers of long standing, went to term and the blood returned to normal within several days postpartum; ewe No. 4, for example, appeared healthy when slaughtered, despite gross fattiness of the liver.

Such data as is available does not suggest that fatty infiltration of the liver occurs in normal, well-fed ewes. Many ewes in the field, however, do not receive an optimum diet and it is probable that ketosis and some degree of fatty infiltration of the liver occurs under these conditions. That such ketosis may be pronounced, though unsuspected by the farmer, is indicated by a subsidiary investigation. Through the courtesy of Sir Joseph Barcroft, samples of blood and liver were obtained from experimental ewes being used at Cambridge. These ewes, presumably in apparent good health, were slaughtered at various stages of gestation, and it was found that after about the 100th day blood from some of the ewes contained ketone bodies, as much as 22 mg./100 c.c. being found. Histological examination of the respective liver samples showed that quite marked fatty infiltration had taken place. Blood samples from other ewes in the same flock (one slaughtered as late as the 146th day of gestation) were quite free of ketone bodies and the livers appeared normal histologically. Variable food consumption by ewes being handled in groups could explain the presence of a certain number of ketonaemic animals in a flock of ewes all carrying the same number of lambs.

SUMMARY

Chemical and histological studies have been made concerning the degree, nature, and distribution of the fat present in the livers of healthy, pregnant laboratory animals, normal ewes (barren and pregnant), ketonaemic ewes, and field cases of pregnancy disease. It is concluded that

Fatty infiltration of the liver is not a normal concomitant of pregnancy in the rat, rabbit, guinea-pig, or sheep.

The fatty infiltration of the liver associated with ketosis in the pregnant ewe does not necessarily provoke harmful symptoms; animals may go to term when the infiltration is extreme.

In ketonaemic, pregnant ewes the glycogen content of the liver is apparently reduced to negligible amounts.

Ketonaemic ewes in apparent good health were found to have livers infiltrated with fat, the nature and degree of the infiltration being indistinguishable from that seen in comatose ewes. The fatty liver produced in the experimental ewes could not be distinguished from that found in field cases of the disease.

Fatty infiltration of the liver was not associated with necrotic changes in the liver tissue.

I wish to thank Mr W. Godden for advice in the chemical work, and Dr J. T. Irving for guidance in the histological work and also for taking the microphotographs. Part of this study was carried out during the tenure of a Hackett Research Studentship from the University of Western Australia.

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EXPLANATION OF PLATE 1

1. $\times 11$. A frozen section stained with Scharlach R and haematoxylin to show distribution of the fat in the liver tissue of a "healthy" ketonaemic ewe (No. 23, Group IV, 1939) with 3 lambs in utero. The liver contained 46 % total fat in the dry matter.
2. $\times 39$. A paraffin section stained with haematoxylin and eosin D showing the extreme fatty infiltration of the liver found in a ketonaemic ewe (No. 4, Group V, 1937-8) slaughtered immediately after lambing (2 lambs). The liver contained 71 % total fat in the dry matter.
3. $\times 39$. A paraffin section stained with haematoxylin and eosin prepared from the liver of a hypoglycaemic ketonaemic ewe (No. 3, Group VI, 1937-8) killed after 2 days' coma (2 lambs in utero). The liver contained 63 % total fat in the dry matter.
4. $\times 67$. As No. 3 at higher magnification.
5. $\times 67$. A paraffin section stained with haematoxylin and eosin prepared from the liver of a healthy experimental ewe slaughtered at lambing because of a torn uterus. The liver contained 25 % total fat in the dry matter.

VASCULAR CHANGES AFFECTING THE
TRANSMISSION OF NERVOUS IMPULSES

BY EDITH BÜLBRING AND J. H. BURN

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WE have recently described experiments in which we studied the effect of sympathetic stimulation on the contractions of skeletal muscle in the dog [Bülbring & Burn, 1939*a*]. To make our observations we exposed the spinal cord and dissected the motor roots, in order that stimuli could be applied to the motor fibres only. We perfused the muscles of the corresponding hindleg with defibrinated blood. In these circumstances we have often observed that the contractions of the gastrocnemius in response to motor root stimulation declined and finally disappeared, but could be restored by various measures, such as the addition of adrenaline to the perfusing blood. We have discovered that the disappearance of the contractions was not due to any change in the muscle or at the nerve ending but to a failure of the motor fibres to transmit the impulses along the sciatic nerve. This paper is an account of these observations.

METHOD

The experimental details have been given in the previous paper and little need be added. As there stated, the blood from the pump entered the right external iliac artery and went via the aorta to the left external iliac artery. The aorta was ligated above and below. There is usually a pair of arteries proceeding dorsally from the aorta, which in our present experiments we have tied to diminish the leakage of blood from the tissues in the neighbourhood of the exposed motor roots.

When the preparation was made in this way, the period in which it was possible to elicit a response to stimulation of the sympathetic chain at a point just below the kidney was very short, for the sympathetic ganglia were deprived of their blood supply and ceased to transmit impulses. In experiments therefore in which sympathetic stimulation

was applied, the aorta was not ligated above the bifurcation but higher, below the origin of the renal arteries, so that some blood passed up the aorta and through the small vessels to the back. This blood caused leakage near the exposed motor roots but mostly found its way into the vena cava from which it was collected by a cannula in the thoracic cava, or in the right auricle.

The essential observations were made not only in experiments in which defibrinated blood was used for the perfusion fluid, but also when heparinized blood was used; the results were the same.

RESULTS

Failure of motor root response. In Fig. 1 is shown a typical example of the changes in the response to motor root stimulation. The stimulation applied was a tetanus of 10 sec. duration, produced by shocks at the rate of 400 per sec. The first stimulation shown in the figure produced a tension in the gastrocnemius of 10 kg., which fell slightly during the stimulation; a similar response had been given to several previous stimulations. During this time the vascular tone in the perfused vessels had remained high due to the presence of adrenaline which had been put into the blood before perfusion began to make a concentration of 1 in 10 million. During the application of the tetanus the middle record, which is of the venous outflow, taken by Gaddum's recorder [1929], showed a rise due to the expulsion of blood when the muscles contracted; the lower record of pressure in the arterial cannula showed an initial rise due to obstruction of inflow, followed by a fall during contraction and a further fall when the muscle relaxed; these vascular effects have been discussed in the preceding paper. The vascular tone now began to fall as the adrenaline was destroyed, and the response to motor root stimulation grew less. These stimuli were applied at intervals of 2 min., and the second, third and fourth responses shown in Fig. 1 were recorded, 6, 21 and 24 min. respectively after the first. The fourth response was already very small, consisting of an initial spike of 2.6 kg. which was not maintained during the 10 sec. of stimulation above 0.7 kg. At this point of the experiment adrenaline of strength 1 in 100,000 was added drop by drop to the reservoir of blood supplying the leg. After 3 min. the adrenaline reached the vessels of the leg, causing vasoconstriction and a reduction in the venous outflow. There was, however, no immediate effect on the height of the muscular response; it was 5 min. later that this first showed itself, but then the recovery was rapid. In 2 min. more the tension was 9 kg. and in 4 min. it was 9.8 kg.; in 6 min. it was as high as at first. The

addition of adrenaline to the reservoir was stopped when a total of 0.1 mg. had been added. The vascular tone then fell, and the response once more fell until it disappeared altogether. The addition of adrenaline to the reservoir was restarted and the response to stimulation once more fully recovered.

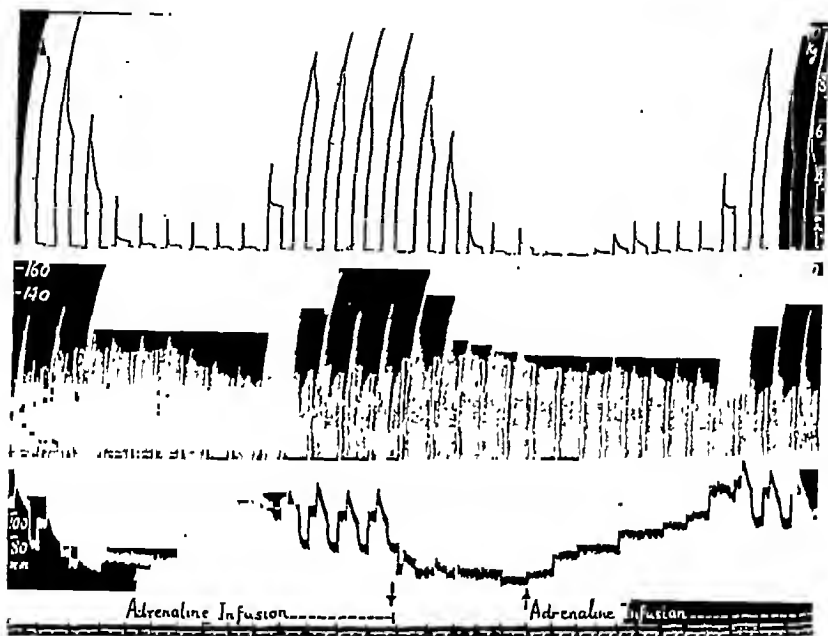


Fig. 1. The upper record shows the tension developed in the gastrocnemius muscle during the application of tetani of 10 sec. duration to the motor roots. The middle tracing shows the venous outflow recorded by Gaddum's recorder. The lower tracing is the pressure in the arterial cannula into which blood is delivered by the Dale-Schuster pump. The figure illustrates the decline of the motor root response following the loss of vascular tone; also the recovery of the response following the rise of tone produced by adrenaline. This decline and recovery are shown twice.

Consideration of the changes in venous outflow shown in Fig. 1 makes it clear that the height of muscular contraction did not vary with the blood flow through the leg, but was rather in inverse relation to the blood flow. That is to say, the contraction failed as the arterial resistance to inflow fell and the outflow increased; similarly the contractions returned as the arterial resistance rose and the outflow diminished. There was, however, a lag between the maximum arterial pressure and the maximum muscular response, for the latter was observed usually about 5 min. later.

Conditions in the muscle. The first possibility which presented itself in explanation of the disappearance of the contractile response was that in the conditions of the perfusion there was a failure of the chemical mechanism in the muscle. Thus the store of muscle glycogen might have become exhausted; with this idea we investigated the effect of adding substances other than adrenaline to the blood which play a role in glycogen formation. We found that the addition of dextrose, or the addition of dextrose and insulin, or the addition of sodium lactate, had no effect in restoring the response. In myasthenia gravis, in which there is muscular weakness, glycine is stated to have some beneficial action, and we therefore added glycine (100 mg. to 600 c.c. blood); it had no action. In Addison's disease, cortical extract increases muscular power. We therefore added Eucortone, but without effect.

Failure of response to single shocks. Evidence that the chemical mechanism in the muscle was not impaired was provided by observations that the contractile response to direct stimulation of the muscle remained unaltered during the disappearance of the response to stimulation of the motor roots. Furthermore it was found that in order to observe this disappearance of response to motor root stimulation, the application of tetanic stimuli was unnecessary, since the response to single shocks, applied at intervals of 10 sec., disappeared in the same way. These points are illustrated in Fig. 2A which shows in the beginning a group of contractions (a) due to six single shocks applied to the motor roots, each producing a tension in the gastrocnemius of about 8 kg. The second group of contractions (b) was obtained 1 min. later in response to single shocks applied to the muscle directly; these produced a smaller tension of 6.5 kg. Groups similar to (a) and (b) had been obtained since the beginning of the perfusion more than 30 min. previously, during which time the vascular tone steadily diminished and the venous outflow increased. Single shocks were now applied to the motor roots only at intervals of 10 sec. The tension developed in the muscle by each stimulus now fell, gradually at first, and then very rapidly, and the last six responses are shown in the third group (c) of Fig. 2A, the sixth stimulus produced a tension of 3 kg. only. The stimuli to the motor roots were stopped, and 1 min. later stimuli to the muscle were applied directly; the effect of these direct stimuli (d) was completely unaltered. After 1 min. more, six further responses (e) to motor root stimulation were obtained, which were very small, and finally six further responses (f) to direct stimulation, again of the same height as before.

Changes in the sciatic nerve. The evidence obtained in this way made it

clear that the disappearance of the response to the motor roots was unconnected with changes in the muscle and, since single shocks were used, was unrelated to any fatigue of the nerve fibres or their endings produced by repeated stimulation. The lack of connexion with fatigue was specially emphasized by one experiment in which the perfusion began some time before the motor roots were ready for stimulation, and in which the vascular tone had already fallen very low before the stimulation was first applied. Single shocks to the motor roots produced no effect



Fig. 2. Records of the tension produced by groups of single shocks at 10 sec. interval applied alternately to the motor roots, and to the gastrocnemius muscle. In A the effect of motor root stimulation was normal at (a), diminishing in (c) and small in (e), while that of direct stimulation at (b), (d) and (f) remained unchanged. In B the addition of adrenaline to the circulating blood restored the motor root stimulation from (a) through (c) to (e). Direct stimulation (b, d, f) was as before unaffected.

whatever, though they soon caused muscular contraction when adrenaline was added to the perfusing blood in the same way as in the experiment illustrated in Fig. 2B.

The next stage of the analysis concerned the sciatic nerve, to discover whether the failure of the motor root stimulation was due to a failure of the nerve endings to transmit the impulse, or to a failure of excitability or conductivity in the nerve fibres themselves. In addition to preparing the motor roots, we applied an electrode to the sciatic nerve between the flexor muscles of the thigh. This electrode consisted of a platinum plate shielded by a curved vulcanite prong. The common electrode placed under the skin of the back was the second electrode. We were then able to

stimulate the motor roots and the sciatic nerve alternately, and a record of an experiment is given in Fig. 3. Single shocks at 10 sec. interval were used. Fig. 3 shows in the beginning (*a*) the regular response to motor root stimulation, and (*b*) the less regular though slightly larger response to stimulation of the sciatic nerve. As seen in (*c*), (*e*), (*g*), (*i*) and (*k*) the responses to motor root stimulation became progressively less until they disappeared, while the responses to sciatic stimulation persisted, showing a slight diminution only in (*f*), (*h*), (*j*) and (*l*). This result indicated that there was no failure of transmission from the nerve to the muscle, but that the failure occurred along the course of the nerve fibres, chiefly near the motor roots but also to a slight extent near the electrode on the sciatic nerve. It seemed as if the decline of vascular tone must in some way have

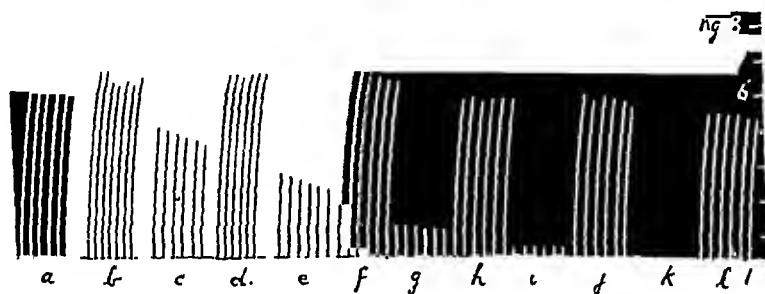


Fig. 3. Records of the tension produced by groups of single shocks at 10 sec. interval applied alternately to the motor roots and to the sciatic nerve. In *a*, *c*, *e*, *g*, *i*, *k*, etc. is shown the gradual diminution and final disappearance of the motor root response, while the sciatic response (*b*, *d*, *f*, *h*, *j*, *l*) remained large, though declining slightly.

caused a diminution in the excitability or conductivity of the motor fibres, which was greatest in the neighbourhood of the roots, where there was most disturbance of the normal circulation, and least in that part of the sciatic nerve nearest the knee. We had not believed that differences in blood supply could modify the behaviour of different parts of continuous nerve fibres, and we tested the point further in experiments of the following kind.

A perfusion of the hindleg muscles was prepared in which the arterial cannula was inserted into the femoral artery in the middle of the thigh, and the blood collected again from the femoral vein. Mass ligatures were tied around the upper part of the thigh, carefully excluding the sciatic nerve which was dissected upwards to the sacral plexus. In this experiment the muscles of the upper part of the thigh had no circulation and could not furnish collaterals to the proximal part of the sciatic nerve.

Only the lower part of this nerve could have been supplied with blood. Stimulation of the motor roots produced no contraction. Stimulation of the sciatic nerve produced a contraction which varied according to the nearness of the electrode to the knee. In Fig. 4 are shown the tensions produced by stimuli applied to different points of the nerve 2 cm. apart; stimulation nearest the plexus had no effect; as the electrode was taken towards the knee the response progressively increased. To ensure that the differences were unconnected with the changes in the position of the electrodes, two electrodes were applied and left undisturbed. Single shocks applied to the proximal electrode produced a tension of 1.5 kg., while when applied to the electrode nearer the knee, they produced a tension of 8 kg.

Effect of adrenaline on the sciatic nerve. By the addition of adrenaline to the circulating blood, in another experiment we were able to affect the conditions in the sciatic nerve at the site of the proximal electrode so that the tension developed in response to single shocks increased from 2.7 to 3.3 kg., while the tension developed by single shocks applied to the electrode near the knee remained unchanged at 8 kg. The addition of the adrenaline caused a rise in arterial pressure from 32 to 120 mm., but diminished the out-flow from 80 to 48 c.c./min. Since there could have been no circulation from the pelvic end of the nerve reaching the point of application of the proximal electrode, the adrenaline must have exerted its effect through such circulation as reached that point from vessels entering the nerve in the neighbourhood of the knee.

This evidence of a partial restoration of the function of the proximal part of the sciatic nerve indicated that we were observing a similar phenomenon to the disappearance and reappearance of the response to motor root stimulation already described.

Alterations of blood flow. The most likely explanation for the failure of the gastrocnemius to respond to stimulation of the roots appeared to be that owing to the fall in arterial pressure the motor fibres near the electrodes were no longer supplied with sufficient blood; their function

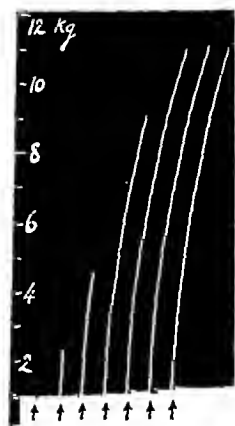


Fig. 4. Records of the tension produced by single shocks applied to different points of the sciatic nerve. Stimulation of the nerve farthest from the muscle was without effect (first arrow). Stimulation at points successively 2 cm. nearer the muscle produced increasing tension until a further approach made no difference.

was restored by adrenaline because the rise of pressure which it caused enabled more blood to reach these fibres. If this explanation were correct an increase in arterial pressure caused by raising the stroke of the pump should have the same restorative effect. We thought this the more likely since we have previously found that when interrupted stimuli are applied

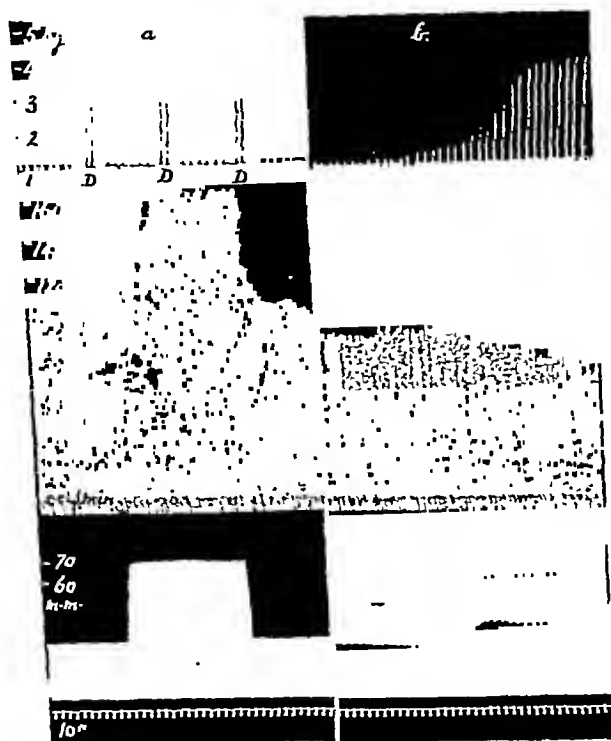


Fig. 5. Records as in Fig. 1. The upper record shows the very small tensions produced by single shocks at 10 sec. interval applied to the motor roots, with three groups of two direct stimuli (*D*). (*a*) shows that a rise of perfusion pressure caused by raising the pump stroke did not affect the response to motor root stimulation. (*b*) shows that the addition of adrenaline restored the motor root response although it did not raise the perfusion pressure higher than in (*a*) and reduced the blood flow.

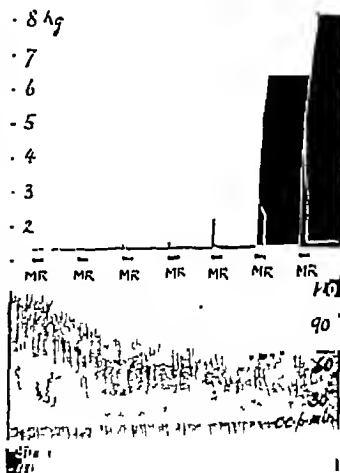
to the sciatic nerve the contractions of the gastrocnemius can be increased by raising the stroke of the pump. We showed [Bülbring & Burn, 1939c] that this effect was exerted in the main on the nerve endings.

We found that an increase in arterial pressure produced by raising the pump stroke did not restore the effect of motor root stimulation. In Fig. 5*a* the arterial pressure was raised from a mean value of 20 mm. to

56 mm. during $2\frac{1}{2}$ min., resulting in an increase of venous outflow from 100 to 166 c.c./min.; the responses to motor root stimulation were unaffected. In Fig. 5b the arterial pressure was raised, by addition of adrenaline to the reservoir, at first to 37 mm., later to 50 mm. and then to 64 mm.; the venous outflow decreased to 70 c.c./min. When the arterial pressure was 50 mm. the responses to motor root stimulation had already increased to 3.6 kg. In other experiments the pump stroke was raised for longer periods, for 12 and 16 min. respectively, and yet the motor root responses were unaffected; in both of these the addition of adrenaline to the perfusing blood at once restored the responses.

Effect of constrictor substances. We were next able to show that in fact the rise of arterial pressure produced by adrenaline played no part whatever in the restorative action, for we prevented the rise of pressure by connecting the arterial cannula by a side-tube to a reservoir of blood under constant pressure [see Kraye & Verney, 1936]. When adrenaline was added to the perfusing blood and vasoconstriction occurred in the leg, there was no rise of pressure because the blood was driven into the reservoir; the venous outflow from the leg was reduced to less than half, but nevertheless the response to motor root stimulation was restored.

We obtained the same effect with pituitary (posterior lobe) extract as shown in Fig. 6. In this experiment the stimuli were tetani of 10 sec. duration applied to the motor roots every minute. At the beginning of the record the stimuli evoked no response; at this time the venous outflow was 120 c.c./min. The arterial cannula was connected to the reservoir of blood in which the pressure was maintained at 27 mm., and a total of 4 units (equivalent to 0.4 c.c. of the usual commercial extract) was added to the perfusing blood, of which there was 600 c.c. in circulation. The



Pituitary Extract Infusion

Fig. 6. Records as in Fig. 1. At MR tetani of 10 sec. duration were applied to the motor roots, producing at first no effect, but later a large contraction. The change was brought about by the addition of pituitary extract to the blood; this caused no appreciable rise of arterial pressure because the arterial cannula was connected to a reservoir of blood at constant pressure. The pituitary extract, however, caused a great reduction of venous outflow.

reservoir of blood did not fulfil its duty completely, for the arterial pressure rose very slightly to 34 mm.; the venous outflow fell to 60 c.c./min., but the response to motor root stimulation was restored.

The amounts of adrenaline and of pituitary extract necessary were similar in different experiments, though we made no attempt to determine the least concentration which had some effect. Usually we added 0.1 mg. adrenaline to 600 c.c. blood, but once restoration of the motor root response was obtained with one-half this amount making a concentration of 1 in 12 million. In experiments with pituitary extract we obtained recovery with 1.5-7.5 units, the average amount being 3.5 units in 600 c.c. blood.

We have not tested any series of sympathomimetic substances but we have found that dihydroxyphenylethylamine restored the motor root response. We added 10 mg. to the circulating blood, and it produced a similar effect to 0.1 mg. adrenaline. The effect of adrenaline was abolished by ergotoxine as was the vasoconstrictor effect also. In one of the three experiments in which ergotoxine had abolished the action of adrenaline, the response to motor root stimulation was restored by pituitary extract.

The action of vasotonins. At an early stage of the investigation we observed that when blood which had been standing in a vessel was added to the circulating blood, an augmentation of the response to motor root stimulation followed, accompanying the vasoconstriction. In a communication to the Physiological Society [Bülbring & Burn, 1939*b*] we stated that the response of the muscle to motor root stimulation was restored "by greatly increasing the blood flow through the muscles". This statement depended on observations made when blood from a reservoir under high pressure was driven through the leg in addition to that coming from the pump. The effect on the muscle response of a sudden increase of blood flow brought about in this way was greatest the first time we tried it in each experiment; successive repetitions produced progressively smaller effects up to a point after which the increase remained the same. We attributed the diminution in successive effects to the disappearance of vasotonins from the blood, and thought that the persistence of a smaller increase after five or six repetitions indicated the effect of the increased blood flow. If, however, as we have already described, the increase of the blood flow is brought about by increasing the stroke of the pump so that only blood in continuous circulation is driven into the leg, then no increase of muscle contractions is observed. We conclude that when blood is withdrawn from circulation and put in a reservoir even for so short a time as 8-10 min. vasotonins can form in it and produce their effect.

The effect of sympathetic stimulation. When the response of the gastrocnemius to stimulation of the motor roots became small we found that stimulation of the lumbar sympathetic chain was able to restore the contractions to their original size. In one experiment we first stimulated the chain when only a very slight diminution of the tension evoked by

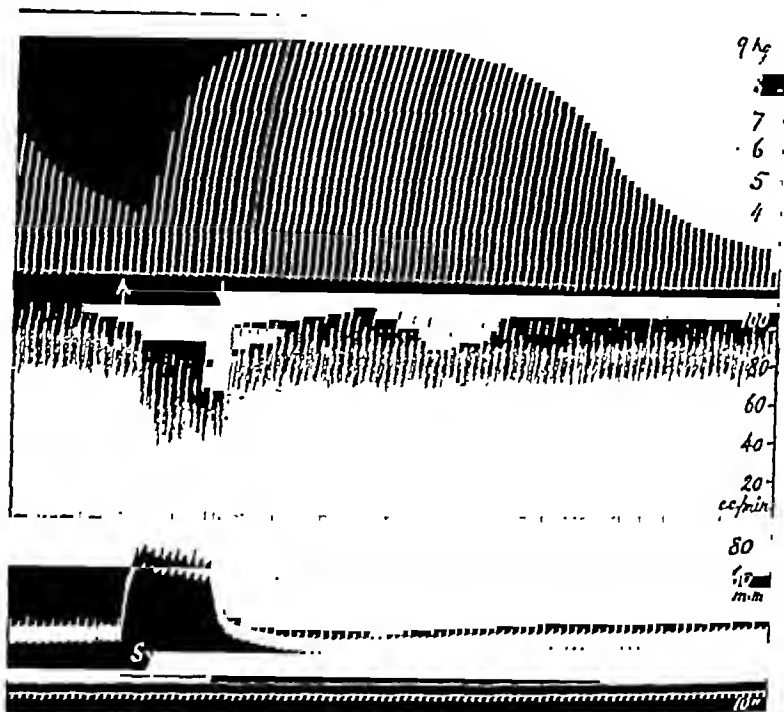


Fig. 7. Records as in Fig. 1. The top record shows the response to single shocks applied to the motor roots. Between the two arrows the lumbar sympathetic chain was stimulated for 2 min. causing a rise of arterial pressure, a diminution of venous outflow and a prolonged increase in the muscular response.

single shocks had occurred. The initial tension was 9.5 kg., and it fell to 9 kg.; stimulation of the chain for 1 min. restored it to 9.5 kg. The next stimulation of the chain was applied when the tension had fallen to 6.5 kg.; it restored the tension to 9.5 kg. The third stimulation of the chain is shown in Fig. 7; it was applied for 2 min. and augmented the tension from 3.7 to 9.2 kg.; this figure shows the much greater duration of the effect of sympathetic stimulation on the muscle response than on the blood vessels; the effect on the muscle lasted for 9 min. whereas that

on the blood vessels disappeared within 30 sec. of the end of the stimulation. A fourth stimulation augmented the muscle response from 2.2 to 8.3 kg., producing an effect similar to that in Fig. 7.

We obtained the same result in eight other experiments when single shocks were applied to the motor roots, and in five other experiments in which tetani were applied. It can be seen in Fig. 7 that the first response after sympathetic stimulation began was slightly smaller than the preceding and succeeding responses. This occurred throughout this experiment, and we observed a diminution only of the response to single

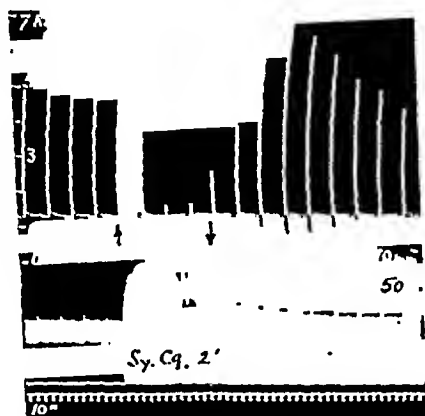


Fig. 8. The upper record shows the response of the gastrocnemius to tetani of 20 sec. duration applied to the motor roots. Between the arrows the lumbar sympathetic chain was stimulated; the stimulation caused first a diminution of the response to the motor root stimulation, followed by an augmentation; there was a rise of arterial pressure, and a diminution in venous outflow (not shown).

shocks as a consequence of sympathetic stimulation in two other experiments. In one of these the diminution was from 7.5 to 7.0 kg. and in the other from 4.0 to 3.8 kg.; that is to say these diminutions were small, and would perhaps have escaped notice but for the observation that, when tetani were applied to the roots, greater effects of this kind were regularly seen. Thus in Fig. 8 we stimulated the motor roots by tetani applied for 20 sec. with intervals of 10 sec. between. The response had already become small, consisting of an initial tension of 5 kg. which fell almost at once to the resting value during the remainder of the tetanus; stimulation of the sympathetic chain for 2 min. then caused a reduction of the initial tension from 5 to 1.5 kg., and an increase in the height of the muscle response only when the stimulation stopped. The same picture

was observed several times in this and other experiments; the diminution alone, without a succeeding augmentation, was observed in one experiment in which the responses to tetani were still large, the reduction produced by sympathetic stimulation being from 8 to 6.5 kg.

DISCUSSION

We have previously shown that when the muscular contractions produced by stimulation of the motor roots in the dog become less, due to fatigue, they can be augmented by sympathetic stimulation. We did not put forward any explanation of this action of the sympathetic beyond saying that the metabolism of the muscle was probably affected in such a way that more energy was made available; thus we conceived the effect to be exerted on the muscle directly. We have since investigated the action of adrenaline on the muscle contractions produced by direct stimulation and by stimulation of the sciatic nerve, and have observed that the augmentation produced when the muscles are fatigued is best seen when the stimulation is through the nerve; during direct stimulation the augmentation is either small or absent. This evidence led us to modify our previous view and to suppose with Orbeli [1923] and Corkill & Tiegs [1933] that the main effect was on the neuromuscular junction rather than on the muscle, though some direct action on the muscle could not be excluded. We have now presented evidence that not only at the nerve ending, but along the course of the nerve fibre itself, adrenaline and sympathetic stimulation can improve the transmission of impulses.

The work of Forbes & Ray [1923] showed that a mammalian nerve removed from the body and kept in Ringer's solution retains a normal functional capacity for a long time, especially if it is kept cold. This observation naturally leads to the assumption that the chemical requirements of nerve fibres are so small that they would be satisfied so long as some circulation is maintained no matter how small this circulation may be. Our evidence indicates that this is not so, and that excitability or conductivity can fail while a blood flow still persists. It is, however, much more surprising to observe the rapidity with which these functions can be restored, and the agents which can restore them. We found that the contractions evoked by single shocks applied to the motor roots at 10 sec. interval were augmented within 20 sec. of the application of a stimulus to the sympathetic chain; on the basis of our other experiments it can be assumed that this augmentation was due to a change in the nerve in the neighbourhood of the roots whereby more fibres were excited or enabled

to conduct impulses, since no corresponding change was observed in the response to sciatic nerve stimulation nearer to the muscle.

The mechanism by which this change was brought about is difficult to understand, but since the change was produced by adrenaline, pituitary extract, vasotonins and sympathetic stimulation, it was apparently the result of vasoconstriction. One can imagine, for example, that the blood supply of nerve trunks, like that of the brain itself, depends on the blood pressure which is produced by the vascular tone in other tissues, and that adrenaline acts by constricting muscle vessels and so deviating more blood into the nerve trunk. Since, however, adrenaline and pituitary extract exerted their action on the nerve when the arterial pressure did not rise, and when vasoconstriction in the muscle vessels merely served to deviate blood into a reservoir at constant pressure, it is difficult to accept this explanation. Moreover, we found it impossible to restore the response by raising the pump stroke although this doubled the blood flow. A second explanation is that adrenaline restored the function by causing vasoconstriction in the nerve trunk vessels themselves. Otherwise it must be concluded that all four agents have some other property in common as well as a vasoconstrictor action, which affects the nerve directly, and that it is through this they produce their effect.

The diminution in the muscular response to single shocks applied to the motor roots caused by sympathetic stimulation has not yet been analysed; it may be due to a change on the course of the nerve fibres or to a change at the nerve ending.

According to our evidence sympathetic impulses or adrenaline can act in three ways on the contractions of skeletal muscle. They have a small action in augmenting the contractions of fully curarized muscle which must be due to an effect on the muscle itself. They have a more important action in improving neuromuscular transmission, probably by augmenting the number of impulses which become effective. Recent evidence of this action has been provided by Bjurstedt & von Euler [1939]. A third action may be exerted in improving the excitability and conductivity of the motor nerve fibres, though at the present it is impossible to say how important this action is when there has been no interference with the natural circulation.

SUMMARY

1. The experiments described show that, in the conditions of a perfusion system, impulses applied to the motor roots fail to be transmitted along the sciatic nerve to the gastrocnemius muscle of the dog

when the vascular tone becomes low. This failure is not due to fatigue and occurs when single shocks are applied. The failure is not at the nerve ending.

2. The transmission of impulses along the nerve can be restored by the addition of constrictor substances to the blood, such as adrenaline or pituitary extract, or by the addition to the circulation of blood containing vasotonins. The transmission of impulses cannot be restored by increasing the perfusion pressure even though this doubles the blood flow.

3. The transmission of impulses can be restored by stimulating the sympathetic chain, and improvement can be observed within 20 sec. of applying such a stimulus. The restoration so produced long outlasts the effect of the stimulation on the blood flow.

We wish to express our thanks to Mr H. W. Ling whose assistance greatly facilitated our work.

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AMINE OXIDASE AND ADRENALINE

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THE amine oxidase has been shown to oxidize adrenaline *in vitro* [Blaschko, Richter & Schlossmann, 1937; Richter, 1937], and it has been suggested that it may be concerned in the physiological inactivation of adrenaline liberated by the post-ganglionic sympathetic nerve endings [Gaddum & Kwiatkowski, 1938]. The experiments hitherto described have, however, been done at adrenaline concentrations of the order of 10^{-3} , and before the physiological significance of this enzyme can be assessed it is necessary to know whether the rate of inactivation of adrenaline is still significant at much lower adrenaline concentrations.

With most enzymes the reaction velocity falls off with decreasing substrate concentration according to the Michaelis relation $v = Vx/(K + x)$; but it is not known that the amine oxidase obeys the Michaelis relation, so that it is not possible to estimate from the existing data what would be the rate of inactivation of adrenaline at lower concentrations. Experiments have therefore been carried out to measure the rate of inactivation of adrenaline by the amine oxidase at adrenaline concentrations down to 10^{-7} , which was the lowest concentration that could be estimated accurately by the methods used.

METHODS OF ESTIMATING ADRENALINE

In the present work it was necessary to estimate small amounts of adrenaline in the presence of relatively large amounts of tissue and, in some experiments, in the presence of ephedrine. Under these conditions the usual physiological methods of estimating adrenaline are not reliable [Bain, Gaunt & Suffolk, 1937] and chemical methods were therefore preferred. The adrenaline was separated from the organic material by

deproteinizing with trichloroacetic acid and adsorbing on aluminium hydroxide as described by Shaw [1938]: it was then estimated colorimetrically. For concentrations down to 10^{-4} the red colour formed on adding iodine [v. Euler, 1933] was found to be very accurate and reliable for colorimetric estimation. For lower concentrations the less accurate but more sensitive method of Shaw was used. Preliminary work showed that the latter method required some modification when used under these conditions. It was found advisable to control the *pH* of the first adsorption by adding bromocresol green and adjusting to a blue-green shade. Cresol red was then used instead of phenolphthalein for the final adsorption. Control experiments showed that ephedrine, octyl alcohol and low concentrations of cyanide did not interfere with the adrenaline estimation.

EFFECT OF ADRENALINE CONCENTRATION ON RATE OF INACTIVATION

Ox liver was used as a source of amine oxidase. A fine suspension was prepared by grinding thoroughly for 20 min. with sand, adding an equal volume of water and pressing through muslin. The suspension was dialysed for 5 hr. in a cellophane tube against running tap water and then diluted to 3 volumes after adding sufficient *M*/1.5 phosphate buffer *pH* 7.3 to bring the final concentration to *M*/15. The amine oxidase did not form a true solution but was associated mainly with the solid cell fragments in the suspension. The amine oxidase activity of the preparation was tested by measuring the oxygen uptake in a Warburg apparatus in the presence of tyramine. As an arbitrary enzyme unit was taken the amount of enzyme that caused an oxygen uptake of 1 c.mm./hr. when shaken in the Warburg apparatus under standard conditions (1.8 ml. enzyme suspension containing *M*/15 phosphate buffer *pH* 7.3, 0.1 ml. *M*/50 HCN, 0.1 ml. *M* semicarbazide and 0.2 ml. *M*/4 tyramine hydrochloride shaken in air at 37°. A control experiment without tyramine was done at the same time and the oxygen uptake in 1 hr. without tyramine subtracted from the uptake with tyramine).

In measuring the rate of inactivation of adrenaline 3–4 ml. of enzyme preparation were shaken with 0.5 ml. adrenaline hydrochloride solution (total volume 5 ml.) in the Warburg apparatus at 37°. The amount of enzyme added was adjusted so that approximately 100 units of amine oxidase/ml. were present. Under these conditions about half of the adrenaline was inactivated in 30 min. The results are given in Fig. 1, in which the percentage inactivation in 30 min. is given for adrenaline concentrations down to 10^{-7} .

There is a considerable scattering of the points, but it can be seen that the rate of inactivation was roughly proportional to the amount of adrenaline present, so that over a considerable range of concentrations the percentage inactivation in a given time was comparatively constant. The system therefore adhered approximately to the Michaelis relation.

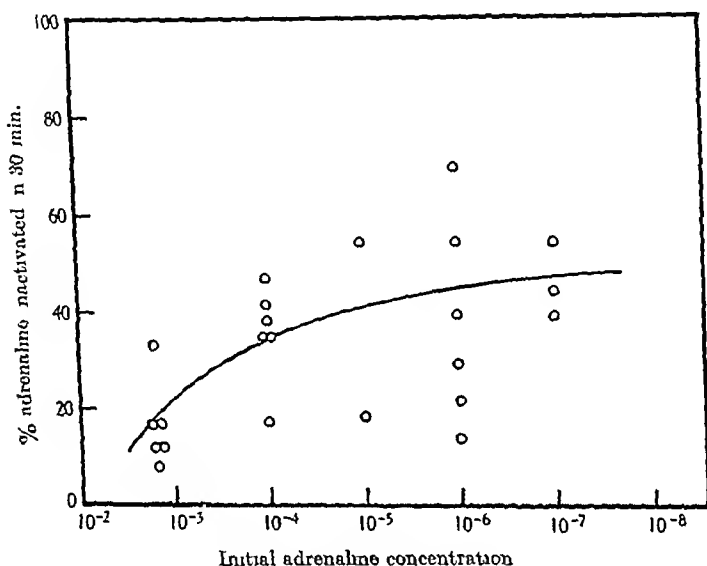


Fig. 1. Inactivation of adrenaline by amine oxidase. (Adrenaline concentrations plotted on logarithmic scale.)

That the amine oxidase was the system mainly responsible for the inactivation at the lower as well as at the higher adrenaline concentrations was confirmed by testing the effect of inhibitors.

TABLE I. Adrenaline (%) recovered after 30 min.

Adrenaline concentrations	Adrenaline alone	HCN 10^{-3}	Octyl alcohol	Ephedrine 10^{-3}
10^{-6}	44	40	77	84
10^{-7}	48	—	76	75

The inactivation was not inhibited by cyanide but was considerably inhibited by saturated octyl alcohol and by 10^{-3} ephedrine: these are the properties of the amine oxidase.

Assuming that the behaviour of the amine oxidase *in vivo* is similar to that *in vitro* it should be possible to calculate how fast adrenaline would be inactivated by the amine oxidase present in the animal body; but

first it is necessary to know the amount of amine oxidase that is present. Bhagvat, Blaschko & Richter [1939] have studied the distribution of amine oxidase in different mammalian organs and have shown that it occurs mainly in the liver. Preparations were made from the livers of a number of different animals, and the amine oxidase activity was estimated by measuring the oxygen uptake with tyramine as described above.

TABLE II. Amine oxidase (units/g. fresh liver)

Omnivorous and carnivorous		Herbivorous	
Cat	264; 220	Guinea-pig	389; 419
Dog	172; 88	Rabbit	177; 242
Pig	186; 336	Sheep	439; 431
Rat	411; 306	Ox	576; 567
Man	261; 358	Tortoise	279
Frog	182		

The experiments with adrenaline at a concentration of 10^{-7} *in vitro* showed that about 50 % was destroyed in 30 min. with a suspension containing 100 amine oxidase units/ml. It can therefore be estimated that in the cat, for example, adrenaline at a concentration of 10^{-7} would be 50 % inactivated in about 12 min. in the liver but much more slowly in the other tissues which contain relatively little amine oxidase. It is clear that the amine oxidase cannot account for the rapid removal of adrenaline from the blood *in vivo* unless the system is many times more active *in vivo* than *in vitro* (Elliott [1905] found e.g. that more than 80 % of 1 mg. adrenaline disappeared from the blood in 3 min. in the cat).

EPHEDRINE AND AMINE OXIDASE

Blaschko, Richter & Schlossmann [1937] observed that ephedrine inhibits the oxidation of adrenaline by amine oxidase, and it has recently been suggested that this may be the basis of the chief pharmacological properties of ephedrine [Gaddum, 1938; Gaddum & Kwiatkowski, 1938]. This action would be comparable with the inhibition of choline esterase by eserine. The original experiments were done with relatively high adrenaline concentrations, and here again it appeared desirable to study the effect of reducing the concentration to values approaching more nearly to the physiological.

Using initial adrenaline concentrations of 10^{-7} , the effect of different concentrations of ephedrine on the rate of inactivation by amine oxidase preparations from ox liver was measured (Fig. 2). The duration of the experiments was 30 min. and the conditions were as described above. With 10^{-3} ephedrine the oxidation of adrenaline was inhibited only 50 %,

and with 10^{-4} ephedrine (i.e. ten times the concentration used in Gaddum and Kwiatkowski's rabbit ear perfusion experiments) the inhibition was already negligible.

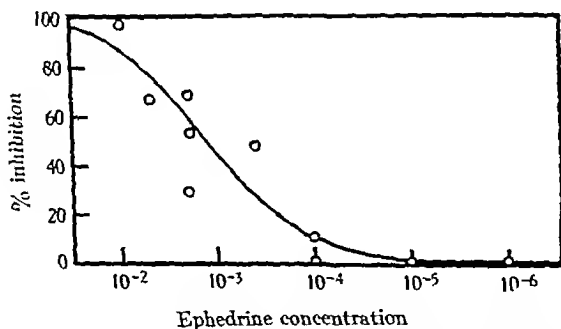


Fig. 2. Inhibition by ephedrine of the oxidation of adrenaline by amine oxidase. Initial adrenaline concentration 10^{-7} . (Ephedrine concentrations plotted on logarithmic scale.)

Amine oxidase in the rabbit's ear. Gaddum & Kwiatkowski, in a very striking experiment, have demonstrated that ephedrine increases the output of "sympathin" after electrical stimulation of the sympathetic nerves of the perfused rabbit's ear. The action of ephedrine is interpreted as being due to its inhibiting the destruction of "adrenaline" by amine oxidase in the ear. Experiments were consequently performed to test for the presence of amine oxidase in the rabbit's ear.

Rabbit's ears were freed from hair and cartilage and cut into fine shreds. On long grinding with sand a tissue suspension of creamy consistency was obtained. The preparation was found to contain no amine oxidase when tested in the usual manner with tyramine as substrate. Bhagvat *et al.* [1939] were also unable to detect any extractable amine oxidase in the rabbit's ear.

DISCUSSION

The amine oxidase has been shown to oxidize a great many amines including adrenaline. That the amine oxidase is also active *in vivo* is indicated by the fact that amines such as isoamylamine and phenylethylamine are rapidly oxidized in the tissues, while amines such as ephedrine and benzedrine, which are not attacked by this enzyme, are slowly excreted unchanged [Richter, 1938]. This suggests that the amine oxidase is probably the system mainly responsible for the detoxication of amines in the body.

It has been suggested that the amine oxidase might be specially concerned in the inactivation of adrenaline or "sympathin" liberated at the post-ganglionic sympathetic nerve endings, but against this view it may be said that (a) adrenaline is not oxidized by the amine oxidase as rapidly as other amines such as isoamylamine and tyramine, which are also of physiological importance, and (b) the present calculations indicate that even in the organs which contain most amine oxidase the rate of oxidation of adrenaline *in vivo* is not likely to be very great; it is doubtful whether the amine oxidase can account for the rapid disappearance of adrenaline from the system which is observed *in vivo*. The distribution of the amine oxidase, which is present mainly in the liver and intestine (and in particularly high concentration in the ruminant) suggests that it is concerned primarily with the detoxication of amines formed by bacterial action in the gut.

The very interesting experiments of Gaddum & Kwiatkowski on the perfused rabbit's ear appeared at first to support the view that the amine oxidase is specially concerned in the physiological inactivation of "sympathin" and that ephedrine works by inhibiting the inactivation; but it is now shown that ephedrine does not inhibit the oxidation of adrenaline appreciably at the concentration used in these experiments, and the rabbit's ear contains little, if any, amine oxidase. In the light of this new evidence there would therefore appear to be difficulties in the way of accepting this view of the action of ephedrine and the function of the amine oxidase.

SUMMARY

1. The rate of inactivation of adrenaline by amine oxidase has been measured at adrenaline concentrations down to 10^{-7} .
2. The degree of inhibition of amine oxidase by ephedrine has been measured using 10^{-7} adrenaline as substrate.
3. The amine oxidase concentration has been measured in the livers of a number of animals.
4. Calculations based on these measurements do not support the view that the amine oxidase is specifically concerned in the inactivation of "sympathin" or adrenaline.

The authors wish to thank Prof. Golla for his interest and the Rockefeller Foundation for supporting this investigation.

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VASCULAR REACTIONS OF THE CAT AFTER TOTAL SYMPATHECTOMY

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THE means whereby an animal deprived of its sympathetic ganglionic chains maintains its vascular tone is still unknown, and there is considerable difference of opinion on the existence of vascular reflexes and the means by which they are mediated in such animals. Bacq, Brouha & Heymans [1934] believe that in the sympathectomized cat vaso-motor nerves, which do not pass through the sympathetic chains, control the vascular reactions of the splanchnic area and maintain the tone of the vessels. Rosenblueth & Cannon [1934] and Pinkston, Partington & Rosenblueth [1936], on the other hand, contend that the dilator fibres in the dorsal spinal roots are probably the efferent paths for the blood-pressure changes which occur in completely sympathectomized cats. All these investigators have insisted on the persistence after sympathectomy of centrally controlled vaso-motor nerves maintaining connexion with the blood vessels. Our own experiments on cats give some support to this conception, but we have been able to demonstrate that many of the vascular reactions, which occur in these animals, can be adequately explained on other grounds.

METHODS

The majority of the operations have been done on female cats weighing between 2 and 3 kg. After subcutaneous injection of 1 mg. of atropine, they were anaesthetized with ether, and a tube was passed between the vocal cords into the trachea, and artificial respiration was started from a pump, with a mixture of air and sufficient ether vapour to maintain anaesthesia. No elaborate preparation of the skin was found necessary, beyond shaving and swabbing with 0.1 % mercuric chloride

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solution. Iodine should not be used for the cat's skin, as it sets up irritation of the wound edges, leading to secondary infection [cf. Liddell & Carleton, 1936]. Our operative technique followed more or less closely that of Cannon, Newton, Bright, Menkin & Moore [1929], with some minor modifications. The most satisfactory procedure in our hands has been the removal at one operation of the whole right sympathetic chain, from and including the stellate ganglion, to the pelvis, together with the abdominal sympathetic chain of the left side from the pelvis to the second lumbar ganglion, the right suprarenal gland being removed also at this first stage. After the cat had recovered from this operation, and had regained its normal weight, the left thoracic chain and the remains of the left upper abdominal chain were removed. In our earlier experiments, at a third operation the remaining, denervated, suprarenal gland was exposed, split along its long axis and the medullary tissue scraped out with a sharp spoon. To ensure more complete destruction of the medulla, the exposed surfaces were touched with a crystal of chromic acid before suturing and returning the gland to its bed. In later experiments, we abandoned this procedure as an unnecessary elaboration, and removed the remaining suprarenal at the time of the final experiment. In point of fact, the presence or absence of the denervated suprarenal has made no detectable difference to our results. In four animals, at the second operation, the semilunar ganglia were excised, and again it may be noted that the reactions of these animals were not different from those of animals in which the ganglia were intact. Miss C. J. Hill has examined histologically portions of the small intestine from three of the cats in which the semilunar ganglion had been excised, and informs us that they contain no sympathetic nerve fibres. The final experiment was done as soon as the cat had recovered completely from the last operation, but in no case earlier than 1 week, or later than 3 weeks after operation. In all cats, except one, the weight at experiment was equal to or greater than the weight before the first operation.

For the final experiment, the cats were anaesthetized with ether and the blood pressure recorded from one carotid artery, the other being tied. They were then decerebrated through a trephine hole, and the vertebral arteries were compressed until bleeding from the transected mid-brain ceased.

Transections of the cord were done by removing the arch of the second cervical vertebra, incising the dura and passing under the spinal cord a thread, the tying of which completed the transection. In those experiments in which nerves were stimulated, the stimuli were either

induction shocks timed by the vibrating hammer, or, more usually, condenser discharges at a frequency controlled by a neon tube device. At the end of each experiment on the sympathectomized animals, a careful post-mortem examination showed that in all cats, with one exception, there were no remaining fragments of the sympathetic chain and that no regeneration had taken place. In the exceptional cat, short lengths of chain including three ganglia on one side and two on the other were found beneath the crura of the diaphragm. It is improbable that these fragments had any significant functional connexions, since the vascular reactions of this animal were in no way different from those in which the sympathectomy was demonstrably complete.

In connexion with experiments on the effects of stimulation of spinal nerve roots, we recorded the temperature of the hairless pads of the cat's hind foot. For this purpose we used an iron-constantan thermocouple of 36 gauge wire. The two wires were soldered together for 2 mm. and the junction so formed was fixed to the surface of the pad with adhesive cellophane tape. The cold junction was kept in ice in a vacuum flask, and the thermal currents were measured with a Moll galvanometer, a balancing current being applied to keep the galvanometer light on its scale. The sensitivity was such that changes of 0.1° C. could be detected with ease.

RESULTS

Effect of decerebration on blood pressure

In four of the sympathectomized cats, the blood pressure was recorded under ether before decerebration. The values observed varied between 80 and 120 mm. Hg from animal to animal. This, in our experience, is significantly lower than the blood pressure of normal cats similarly recorded. Decerebration has little effect on the resting blood-pressure level of the sympathectomized animal; any difference has been in the direction of increase. The pressures recorded after decerebration varied in different animals between 88 and 130 mm., values which are significantly lower than those found in normal cats after decerebration, in which the range has been 120–150 mm.

Vasomotor reflexes after decerebration

(a) *Effect of brief occlusion of the vertebral arteries.*

In the normal decerebrated cat occlusion of the vertebral arteries evokes a steep rise of blood pressure to a level some 80–100% above its previous value. The heart rhythm is slowed during the first few seconds

of the rise and then accelerates. Removal of the clamp on the vertebral arteries is followed by a prompt return to the resting level, with occasionally a little overswing below (Fig. 1A). Removal of the suprarenals may reduce the extent of the rise of pressure, and accelerate the return of pressure to its normal level, but the general character of the effect is unaltered. After section of the vagi, the typical slowing of the heart is absent and the rise of blood pressure may be even more pronounced than in the cat with these nerves intact, and its return to its resting value less steep.

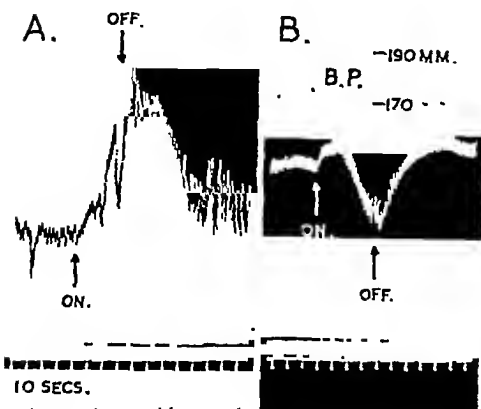


Fig. 1.

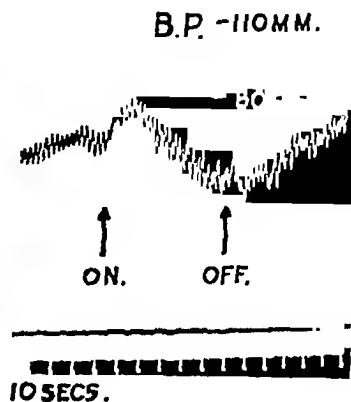


Fig. 2.

Fig. 1. Effect on blood pressure of occlusion of vertebral arteries. A, "normal" cat. B, cat, 2 weeks after sympathectomy.

Fig. 2. Effect on blood pressure of occlusion of vertebral arteries of cat 3 weeks after sympathectomy; both vagi cut.

The response of the sympathectomized cat to the same procedures is constantly the reverse of that described above. Tightening of the clamp causes a small and transient increase of blood pressure, but not more than can be accounted for by the further restriction thus caused of the blood flow to the head of the animal, and this quickly gives way to a fall, accompanied by a pronounced bradycardia (Fig. 1B). As might be expected, section of both vagi abolishes the slowing of the heart and reduces the degree of the depression of the blood pressure (Fig. 2). It is evident, however, that the fall of blood pressure is not entirely due to the vagal effect on the heart. In both the normal and the sympathectomized animal, vertebral clamping produces an extreme hyperpnoea and some muscular movements.

(b) *Effect of stimulation of sensory nerves.*

In the decerebrate normal cat, stimulation of the central end of the cut tibial or peroneal nerves produces widespread somatic reflex movements, with hyperpnoea, if the stimulus is of sufficient intensity, and a rise in blood pressure (Fig. 3A). In the sympathectomized cat, the somatic effects are identical, but the vascular response is always predominantly depressor (Figs. 3B, 7). The small initial pressor effect seen

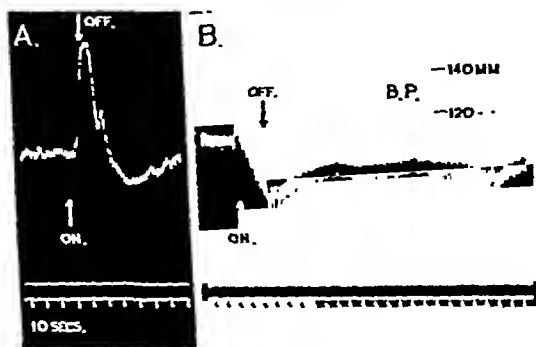


Fig. 3. Effect on blood pressure of stimulation of central end of cut tibial nerve. A, "normal" cat. B, cat, 2 weeks after sympathectomy.

in these figures we believe to be attributable to contraction of the abdominal muscles. Section of the vagi does not alter the depressor effect of sensory stimulation (Fig. 7A).

(c) *Effect of section of the spinal cord.*

As described under methods, the procedure for section of the spinal cord, which we have used, involves exposure of the cord at the level of the atlas, opening of the dura mater, the passage of a thread beneath the cord, and, finally, its section by tying the thread. Under the conditions of our experiments, in which the cord section was carried out on cats previously decerebrated and consequently freed from anaesthetic, the exposure, and, particularly, passage of the thread, was usually accompanied by considerable muscular movement.

Normal cats. In the normal cat, the preparation of the cord and its section evoked big rises of blood pressure, which were followed by a profound fall as the section became effective. That the rises of blood pressure, coincident with exposure and manipulation of the cord, are attributable to the excitation of efferent vasoconstrictor pathways in the cord is

made clear by the fact that they are absent when ether, in an amount sufficient to abolish decerebrate rigidity, is given after decerebration, and that they still are present when muscular movement is abolished by curarine (*vide infra*).

Sympathectomized cats. Entirely different effects follow exposure and section of the cord in the sympathectomized cat. In one experiment we were able to expose the cord and pass the ligature beneath it without

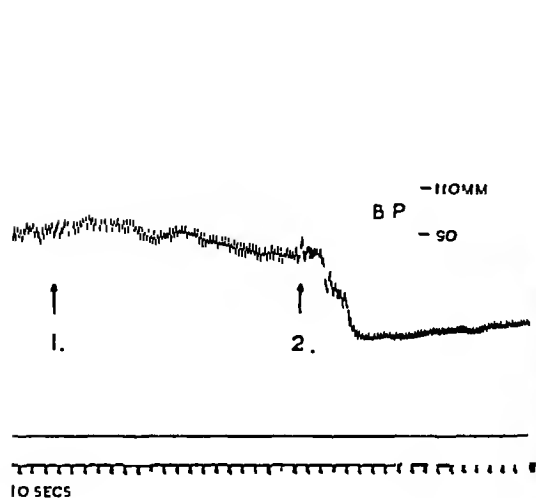


Fig. 4.

Fig. 4. Blood pressure of cat 2½ weeks after sympathectomy. At (1) thread passed under spinal cord; at (2) spinal cord transected.

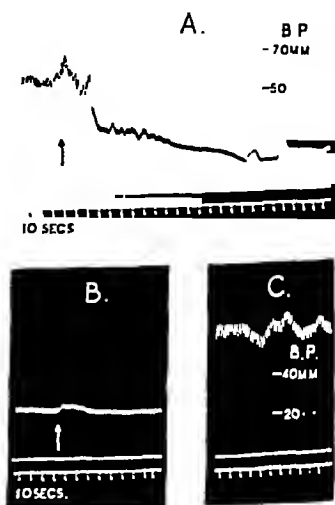


Fig. 5.

Fig. 5. Blood pressure of cat 3 weeks after sympathectomy. A, effect of struggles produced by exposure of spinal cord. B, transection of cord 15 min. later. C, recovery of blood pressure 1 hr. later.

producing any irritation and consequent muscular movement. The blood pressure remained steady. Subsequent section of the cord produced an uncomplicated fall of blood pressure (Fig. 4). This experiment strongly suggested, at the time, that the section had inactivated some unknown pathway which had been maintaining vaso-motor tone. Our subsequent experiments have, however, shown that this deduction is not necessarily true. In all experiments, in which exposure of the cord caused muscular convulsions, a profound and lasting fall of blood pressure occurred, irrespective of whether the cord was cut or not (Fig. 5A). If the cord is cut at the depth of the fall of pressure, there is no further fall, and the

blood pressure gradually returns to the level at which it stood before the exposure of the cord (Fig. 5B, C). In one experiment in which exposure of the cord had produced a severe fall of pressure, the animal was allowed to rest, and some recovery of vascular tone took place. The cord was then frozen with CO₂ snow and cut while frozen. There were no muscular movements and no change in blood pressure. These experiments suggested that the main cause of the depressor responses in the sympathectomized cat might be the vaso-dilatation coincident with contraction of the voluntary muscles. We therefore investigated the responses to cord section, to clamping the vertebral arteries, and to sensory nerve stimulation in decerebrated normal and sympathectomized cats after administration of curarine.

Effect of curarine

(a) *Brief occlusion of vertebral arteries.* Full curarization of the normal decerebrated cat does not alter the vascular effects of brief occlusion of the vertebral arteries. (See p. 275.)

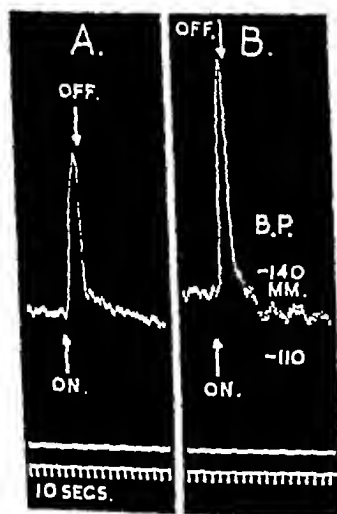


Fig. 6.

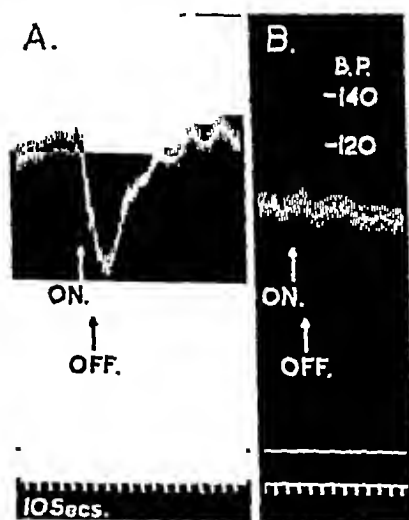


Fig. 7.

Fig. 6. Blood pressure of "normal" cat. Effect of stimulation of central end of cut tibial nerve. A, before; and B, after intravenous injection of 3 mg. of curarine.

Fig. 7. Effect on blood pressure of cat 3 weeks after sympathectomy, of stimulation of central end of cut tibial nerve, both vagi cut. A, before; and B, after intravenous injection of 3 mg. of curarine.

In the sympathectomized cat, the small fall of blood pressure, persisting in the response to vertebral clamping after bilateral vagotomy,

is no longer seen when the animal is curarized. Instead, there is a slight rise of blood pressure, so small indeed that it is impossible to attribute it with certainty to any cause other than the mere restriction of the circulation.

(b) *Sensory stimulation.* The production of complete neuromuscular block by curarine does not diminish the pressor responses of normal decerebrated cats to sensory nerve stimulation (Fig. 6). In the sympathectomized cat, the usual depressor effect of sensory stimulation is completely absent after curarine, and no change in blood pressure occurs (Fig. 7).

(c) *Cord section.* Section of the spinal cord of the decerebrated and curarized "normal" cat reproduced the characteristic rise and subsequent fall of blood pressure, which follow cord section in the cat without curare.

In the sympathectomized cat, on the other hand, curarine modifies profoundly the effect of cord section. In our earlier experiments we found that the usual depressor effects of irritation and section of the cord were absent after curarine¹, but in these animals the blood-pressure level at which cord section was performed was low (40-70 mm.), and this naturally suggested that any hypothetical vaso-constrictor mechanism was already out of action, and that section of the spinal cord could not, therefore, produce any further fall. This low blood pressure was usually the result of too rapid administration of curarine, which has by itself a vaso-dilator action, more conspicuous, perhaps, in sympathectomized animals. In two experiments, therefore, we took the precaution of administering the curarine in 0.25 mg. doses, spread over some 15 min., early in the experiment, removing the remaining suprarenal gland only when the animal was immobilized.

In one of these experiments, the blood pressure under curarine was 130 mm. Hg before the vertebrae were exposed. Removal of the bony arch of the second cervical vertebra was accompanied by a moderately sharp fall of blood pressure to 50 mm. Hg¹. We then completed the exposure of the spinal cord and allowed the animal to rest for 20 min. The blood pressure gradually rose to 100 mm. Hg, and we then passed the thread under the cord and divided it. The blood pressure fell gradually to 70 mm. None of these procedures was accompanied by any muscular movements whatsoever. In the other experiment¹, the blood pressure was 90 mm. Hg after curarine had been given, but before exposure

¹ The three figures illustrating these points were lost after this paper had been received by the Editorial Board. The Board regrets this loss and the consequent delay in the publication of this paper.

of the cord. The neural arch of the vertebra was removed and the dura incised without any immediate change in blood pressure; then the blood pressure suddenly declined to 50 mm. without any new procedure or any other explanation for such a fall. After a rest of 20 min., the blood pressure had recovered to 70 mm., and section of the spinal cord then produced no change.

These experiments suggested the existence of some delicate vaso-motor mechanism, in part responsible for vascular tone and dependent on the maintained functional connexion of the spinal cord with the medullary centres. The spontaneous recovery and maintenance of the blood pressure after cord section (cf. Fig. 5) shows, however, that this is not the sole factor involved. In any case, the relatively crude experiment of section of the spinal cord was obviously not suitable for demonstration of such a system, and we therefore attempted to modify medullary activity by other means.

Effect of prolonged occlusion of the vertebral arteries

Normal cats. The cats were decerebrated, fully curarized and under artificial respiration. We have already noted the immediate effects of clamping the vertebral arteries. If the clamp is left in position for some 15 min., the blood pressure gradually declines from its high level, but remains not much below its height before clamping. During this period, stimulation of the central end of one vagus is ineffective, i.e. one must regard the medulla as being at least partially paralysed by the ischaemia. When the clamp is loosened, the blood pressure returns to its normal level, and the depressor reflex can again be elicited. A renewed application of the clamp, however, evokes, not a sudden rise of blood pressure as it does on the first application, but an abrupt and permanent fall. Stimulation of the central end of the vagus is then permanently without effect. It is obvious that the second clamping has caused a lasting paralysis of the medullary centres.

Sympathectomized cats. The conditions were the same as for the normal cats. The first application of the vertebral clamp (for 15 min.) had no lasting effect on the blood pressure, apart from the small initial rise, which we have already described. The second clamping produced, after a latency of 7 min., a gradual, but profound and permanent fall of blood pressure. In these experiments both vagus nerves had been previously cut in the neck.

Effects of stimulation of anterior roots

The almost complete abolition by curarine of vaso-motor response in the sympathectomized cats had naturally suggested that the depressor effects of sensory stimulation and similar procedures were caused by muscular movements and the consequent vaso-dilatation in the muscles. We have, therefore, recorded the effects on the general blood pressure of stimulation of the anterior lumbar spinal roots in normal and in sympathectomized cats.

Normal cats. In the normal cat, the immediate effect of stimulation of the anterior roots at a tetanizing frequency (50 per sec.) is a rise of blood pressure. As the stimulation is continued, the blood pressure

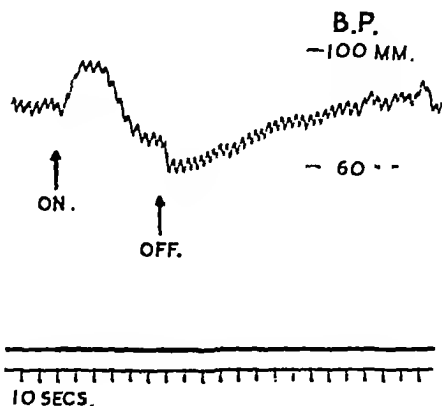


Fig. 8. Effect on blood pressure of "normal" cat of stimulation of anterior spinal roots (sixth and seventh post-thoracic).

returns towards normal, and, at the end of a stimulation lasting a minute, it may even be lower than the resting level. Cessation of the stimulation is, in any case, accompanied by an abrupt, though not large, further fall in pressure and a gradual recovery (Fig. 8). The recovery of blood pressure is complete in from 2 to 3 min. With a stimulation of shorter duration, the entire effect during stimulation is pressor, but the sudden drop of pressure on stopping the stimulus is still in evidence, and is quickly followed by a compensatory rebound, which carries the blood pressure to a level as high as it attained during the stimulation.

Sympathectomized cats. Stimulation of the anterior roots in the sympathectomized cat produces an initial rise in blood pressure of a few

seconds' duration, succeeded by a steep fall, which continues after the cessation of stimulation. A recovery follows, but it is so gradual that the initial level of blood pressure is not attained until after 10–15 min. (Fig. 9A). The administration of curarine, in a dose sufficient completely to abolish the visible response of the muscles to motor nerve stimulation, entirely prevents the fall of blood pressure evoked by excitation of the

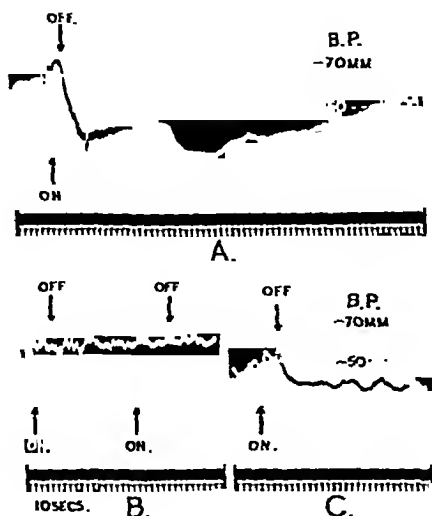


Fig. 9. Blood pressure of cat 2 weeks after sympathectomy. A, stimulation of anterior spinal roots (sixth and seventh post-thoracic). B, stimulation of anterior roots after 2 mg. curarine. C, direct stimulation of leg muscles 32 min. later.

anterior roots (Fig. 9B). Under these conditions, however, the production of muscular movements by direct stimulation of the limb muscles, through electrodes buried in them, is still accompanied by a fall of blood pressure (Fig. 9C).

Stimulation of dorsal roots

Rosenblueth & Cannon [1934] suggested that the abolition of depressor vascular reflexes in the sympathectomized cat by curare was due to the paralyzing effect of curare upon dorsal root dilator fibres. In a later paper from the same laboratory, Pinkston *et al.* [1936] state that curare abolishes vaso-motor reflexes in sympathectomized cats, and this is taken as evidence that "the vaso-dilator fibres in question are the dorsal root dilators". As evidence that curare abolishes the effects of dorsal root stimulation, Rosenblueth & Cannon [1934] refer to Bayliss [1923]. We

have been unable to discover any reference by Bayliss [1923] to such an action of curare; on the contrary, Bayliss [1900] makes it quite clear that the effects of antidromic excitation can be obtained in fully curarized animals, provided the initial vaso-dilatation normally produced by curare is allowed to pass off. (See also Langley, 1923.)

We ourselves have been able to demonstrate that antidromic vaso-dilatation persists in the cat paralysed with curarine. Changes in the cutaneous temperature of the pad of the hind paw of the cat were used as an index of the state of the tone of the vessels, in the manner described by Wybauw [1936]. The greatest rises in pad temperature were obtained by stimulation of the seventh post-thoracic dorsal root (Fig. 10). The administration of the first dose of 1 mg. of curarine caused a rise in pad temperature of 2.5°C .—a rise considerably greater than any we have ever produced by stimulation. This dose was sufficient to block neuro-muscular conduction, but stimulation of the posterior roots still produced a rise in temperature of 0.9°C ., quite definite, though somewhat less than that observed in the same experiment before curarine (1.4°C). A second dose of 1 mg. of curarine produced a rise of temperature of about 1°C . and, when the temperature had again fallen, stimulation still evoked a rise of 0.7°C . (Fig. 10). In any case, apart from the diminished response of vessels already partially dilated by curarine, a diminution in the effectiveness of successive stimulations is to be expected, for Wybauw has reported, and we ourselves have observed, that the response in the cat without curarine progressively decreases and finally disappears.

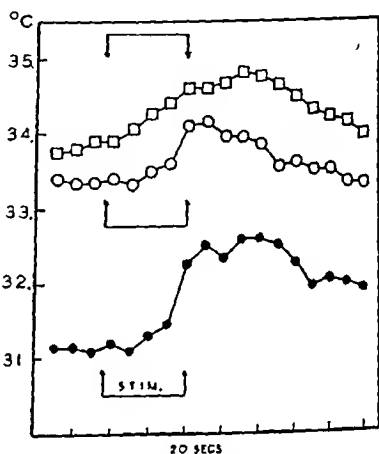


Fig. 10. Effect of stimulation of posterior roots on pad temperature. • Before curarine; □ after 1 mg. curarine; ○ after a further 1 mg. curarine.

DISCUSSION

The primary object of these experiments on animals deprived of their sympathetic chains has been to determine the means whereby such animals regulate and maintain their vascular tone. Ample evidence is available from previous investigations to show that the sympathectomized animal has a resting blood pressure little different from the normal, and

there is no doubt that blood vessels, whether normal, or deprived by degeneration of their sympathetic innervation, can develop and maintain a spontaneous tone. The vessels of the extremities perfused with blood [Burn & Dale, 1926], or haemoglobin solution [Brown & Dale, 1936], react normally to vaso-dilator substances like histamine and acetylcholine, and our own experience shows that this applies to the reactions of the perfused hind limbs of the completely sympathectomized cat, in which the tone of the vessels recovers with normal promptitude from the dilator effect of a small dose of histamine or acetylcholine. Indeed, the depressor effects of these substances retain their normal evanescence in the intact circulation of the sympathectomized cat.

As our aim in this investigation was a study of the vaso-motor activity of the central nervous system, we have tried to use preparations in which that activity was as little depressed as possible, and for that reason we have used decerebrate animals, both for our control experiments, and for the final experiment on the cat after the sympathectomy has been completed.

Bacq *et al.* [1934] have claimed that occlusion of the carotid arteries in the totally sympathectomized cat causes a reflex arterial hypertension of 40–50 mm. Hg. Pinkston *et al.* [1936] and Thomas & Brooks [1935, 1937] have been unable to obtain similar results, but Bacq, Bremer, Brouha & Heymans [1937, 1939] have repeated their original experiments with some modifications of technique, and have been able to confirm the original findings of Bacq *et al.* [1934]. We have not attempted to repeat the experiments of Bacq *et al.* [1934]; indeed, the ligation of the carotid arteries, which we have always carried out as a preliminary to decerebration, has precluded any investigation of carotid sinus reflexes. Under these conditions, we have never observed a genuine pressor response of any sort to any form of stimulation. We have admittedly recorded many small rises of blood pressure of a few mm. in extent and lasting a few seconds (Figs. 3, 9), but in all instances these have been immediately coincident with the onset of somatic muscular movements, e.g. of limbs, of the abdominal wall, or of the respiratory musculature. We are inclined to ascribe these small and evanescent pressor changes to the mechanical disturbances so produced in the vascular bed, and to credit them with no significance as indications of vaso-constriction.

Undoubtedly, in the absence of the carotid sinuses the most predominant vascular response of the sympathectomized cat to any stimulus is a fall of blood pressure. Brief occlusion of the vertebral arteries and sensory nerve stimulation both cause a fall of blood pressure, and this fall is, in both instances, abolished by curarization of the animal.

Stimulation of the anterior spinal roots in the sympathectomized animal produces a similar fall of blood pressure, and this again is abolished by curarization. These observations strongly suggest that the depressor responses, which are so common in the sympathectomized cats, are all attributable to the vaso-dilatation accompanying and consequent on muscular movement. In this assumption we are, in the main, in agreement with Bacq *et al.* [1934], in that they attribute the fall of blood pressure produced by struggling to the peripheral action of muscular metabolites. Rosenblueth & Cannon [1934], Freeman & Rosenblueth [1932] and Pinkston *et al.* [1936], on the other hand, do not believe that muscular metabolites play more than a minor part in the production of such falls of blood pressure. They postulate the existence of a vaso-dilator system with a centre in the medulla and a peripheral pathway *via* the posterior roots, changes in the "tone" of which mediate dilator and constrictor reflexes in sympathectomized cats. We ourselves have been unable to obtain any evidence of the existence of such a mechanism. The conception of the existence of these fibres is based by Pinkston *et al.* [1936] on the abolition of the vaso-dilator reaction by curare and the presence of "depressor points" in the floor of the fourth ventricle. As we have shown experimentally, antidromic vaso-dilatation can occur readily in the cat under curarine, and in any case it is difficult to conceive of any point in the posterior root pathway at which curarine could act. Curarine, suitably applied, has a paralysing effect upon sympathetic ganglion cells [Brown & Feldberg, 1936], but the posterior root ganglion cells are not synaptic and are entirely resistant to the action of nicotine, even when they lie directly in the path of the nerve impulse, as in the skate [Langley, 1901]. Any action of curarine in abolishing vaso-dilator activity could only, if Pinkston *et al.* [1936] were correct, be attributed to a central action. The only evidence that curarine has such a central action is the finding of Rosenblueth & Cannon [1934] that "curare causes a rise of blood pressure in sympathectomized cats". Purified curarine chloride, on the other hand, has, in our experience, always produced a fall of blood pressure in both normal and sympathectomized cats, unless it is administered very slowly, when the blood pressure is unaffected. Similarly, spinal anaesthesia and spinal transection might be expected to cause a rise of blood pressure through the interruption of the vaso-dilator pathways. Bacq *et al.* [1934] and Bradshaw [1936] record respectively a fall of pressure and no effect following spinal anaesthesia, and all authors agree that spinal transection, in the absence of curare, causes a fall of blood pressure.

It is in the interpretation of the effects of spinal transection that we have encountered most difficulty. Most of our evidence points clearly to the fact that the profound and sudden fall of blood pressure, which results from irritation or section of the spinal cord in the sympathectomized cat, is attributable largely to the vaso-dilatation consequent on muscular movement. Nevertheless, exposure of the spinal cord, its section, or ischaemia of the medullary centres, may cause a fall of blood pressure in the sympathectomized cat, even after section of the vagi and full curarization. It might be suggested that such a fall of blood pressure, persisting after full curarization, might still be attributable to motor-nerve impulses, which have been shown to liberate acetylcholine at the motor-nerve endings, even in full curarization [Dale, Feldberg & Vogt, 1936]. These authors have shown that this acetylcholine may diffuse sufficiently to have a local vaso-dilator action, but only in the presence of eserine. In any case, in our own experiments the very considerable falls of blood pressure evoked by direct anterior-root stimulation were completely eliminated by curarization. This seems effectively to exclude not only acetylcholine, but any other vaso-dilator substance, if such were liberated by motor-nerve impulses in the absence of muscular contractions from participation in these falls of blood pressure. We can see no alternative to the conclusion that this fall of blood pressure, following exposure or section of the cord under curarine, is due to the interruption or disorganization of a central vaso-constrictor tone, the effect of which is conveyed to the periphery by some extra-sympathetic path.

Whatever this may be, it is a delicate and sensitive mechanism, which is in action only when the blood pressure is in the region of 100 mm. Hg. In postulating such an unknown pathway, we are in agreement with Bacq *et al.* [1934], who invoked the existence of such a system to explain the occurrence of carotid sinus reflexes, and who believe that its presence can only be demonstrated when the resting blood pressure is at least 100 mm. Hg. We are further investigating its nature.

Whatever may be the functional significance of such a pathway, our experiments have at least shown that its vaso-constrictor activities are readily overpowered by the vaso-dilatation produced by muscular movement, and that this latter factor must be rigidly controlled in any experiments on sympathectomized cats. Indeed, it is the lability of their blood pressure, when they are in any state other than full curarization, which complicates any investigation of the control of the vascular tone of these animals.

SUMMARY

1. The vascular reactions of the cat have been investigated after extirpation of the sympathetic chains.

2. Stimulation of sensory nerves and of anterior spinal roots, brief occlusion of the vertebral arteries and irritation or transection of the spinal cord, produce falls of blood pressure.

3. Abolition of the coincident muscular movements by curarine prevents the occurrence of these falls of blood pressure wholly, in the instance of sensory nerve, or anterior root stimulation, and brief vertebral occlusion.

4. Curarine also abolishes the depressor effect of spinal irritation or transection when the blood pressure is low.

5. If the blood pressure is high, manipulation or transection of the spinal cord causes a fall of blood pressure, even if the cat is immobilized completely by curarine. This suggests the existence of a vaso-constrictor pathway other than the thoracico-lumbar outflow.

6. The predominant factor in the vascular reactions of the sympathectomized cat is, nevertheless, the vaso-dilatation accompanying contraction of the skeletal muscles.

We wish to thank Sir Henry Dale for his help in this investigation.

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THE SYMPATHOMIMETIC ACTION OF LOCAL ANAESTHETICS

BY JEAN TRIPOD

From the Department of Pharmacology, Oxford

(Received 7 July 1939)

IN a recent paper, Gaddum [1938] has put forward a hypothesis concerning the action of ephedrine which can also be applied to other substances. The hypothesis is that ephedrine may exert an effect in one of three ways: it can attach itself to sympathetic receptors and so exert an adrenaline-like action directly; it can attach itself to some of the molecules of enzyme which destroy adrenaline, and thereby increase or prolong the effect of adrenaline; finally, by its attachment to sympathetic receptors of the cells it can prevent the access of adrenaline and thereby diminish adrenaline effects. Macgregor [1939] has recently adopted this hypothesis of the action of ephedrine to explain the effects of cocaine, which since the observations of Limbourg [1892] has been believed to act in some relation to the sympathetic system. Macgregor found that procaine also had various actions which were explicable in the same way. In order to examine further the suggestion which naturally arose from this work that local anaesthetics in general might have some degree of sympathomimetic effect, three other substances, butyn (*p*-aminobenzoylbutylaminopropanol sulphate), percaine (hydrochloride of α -butyloxyeinchoninate of diethylethylenediamide), and stovaine (benzoyldimethylaminoisopropanol hydrochloride) have now been examined, and an account of their action is presented in this paper.

These substances were selected by chance from among well-known local anaesthetics; the toxicity of percaine is greatest, being at least five times that of butyn; the toxicity of stovaine is the least, being about two-thirds that of butyn. Their respective relations to sympathomimetic amines have not been examined, so far as I know, except upon the nictitating membrane of the cat; in this Bacq [1934] found that stovaine augmented the action of adrenaline and tyramine, but that

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butyn and percaïne were without effect. I have examined both the direct action of each of these substances and the effect on the action of adrenaline on the isolated intestine of the rabbit, isolated uterus of the virgin cat, isolated auricles of the rabbit, isolated heart of the cat, the spinal cat for pressor action, the spleen volume *in situ* and on the nictitating membrane of the cat. Adrenaline was used as the *Liquor Adrenalinae Hydrochloridi* of the British Pharmacopoeia, which was diluted in Locke-Ringer; tyramine was weighed in the form of the acid phosphate, the weight of base being taken as two-thirds of that of the salt. The local anaesthetics were also dissolved in Locke-Ringer up to 0.1 %; stronger solutions were prepared in distilled water.

RESULTS

Isolated intestine. A piece of duodenum taken from a freshly killed rabbit was suspended in a bath of oxygenated Tyrode at 38° C. It was found that, like adrenaline, butyn, percaïne and stovaine inhibited the

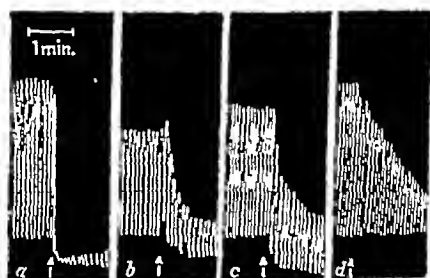


Fig. 1. Isolated duodenum of rabbit in a bath of 60 c.c., showing inhibitory action of (a) 0.015 mg. adrenaline, (b) 0.5 mg. stovaine, (c) 1.0 mg. butyn, and (d) 2.0 mg. percaïne.

intestinal contractions to an extent depending on the concentration. Thus in one experiment a concentration of butyn in the bath equivalent to 1 : 15,000 produced complete inhibition, while a concentration of 1 : 120,000 diminished the amplitude of the contractions by one-half. The activity of percaïne was greater, for complete inhibition was produced by 1 : 40,000 and some diminution by 1 : 600,000. Stovaine also produced complete inhibition in 1 : 30,000 and some inhibitory effect in 1 : 300,000. These actions are illustrated in Fig. 1; it should be noticed that Frugoni [1935] has already described the inhibitory effects of percaïne and stovaine. None of these three substances had any clear effect on the inhibitory action of adrenaline in these experiments.

Isolated uterus. One horn of the uterus of a virgin cat, killed by coal gas, was suspended in a bath of Locke's solution at 37° C. Three of the experiments are illustrated in Fig. 2; thus (a) shows the abolition of tone produced by adrenaline 1:60 million, while (b) shows the inhibition produced by 1:30,000 butyn. The records (c) and (d) were taken from a different cat; (c) shows the effect of the same concentration of adrenaline as before, and (d) the effect of 1:30,000 percaine. In (f) is shown the effect of 1:30,000 stovaine in comparison with that of 1:60 million adrenaline seen in (e); (e) and (f) were taken from a third cat.

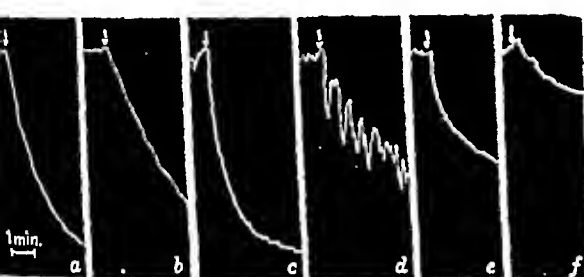


Fig. 2.

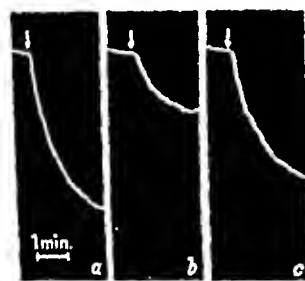


Fig. 3.

Fig. 2. Isolated uterine horn of virgin cat in bath of 60 c.c. The records (a) and (b) were taken from one experiment. (c) and (d) from a second and (e) and (f) from a third. (b) shows the inhibitory effect of 2.0 mg. butyn compared with that of 0.001 mg. adrenaline in (a). (d) is the effect of 2 mg. percaine compared with 0.001 mg. adrenaline in (c); (f) is the effect of 2.0 mg. stovaine compared with 0.001 mg. adrenaline in (e).

Fig. 3. Isolated uterine horn of virgin cat showing diminished inhibitory action of 0.001 mg. adrenaline (b) when 1.0 mg. stovaine had been added to the bath 3 min. before. (a) shows the effect of the same dose of adrenaline before the addition of stovaine and (c) the effect 15 min. after the removal of the stovaine.

All three substances regularly diminished the inhibitory action of adrenaline, as shown for stovaine in Fig. 3. The small effect seen in Fig. 3 (b) was obtained 3 min. after the addition of 1 mg. stovaine to the bath; after repeated washing the large inhibitory effect seen before the stovaine was added once more returned as shown in Fig. 3 (c). The same reversible diminution of the adrenaline inhibition was produced by percaine and butyn.

Isolated auricles of rabbit. The isolated auricles of the rabbit have been suspended in a bath of Locke-Ringer and their contractions recorded with a light lever. To obtain successful results it was important to provide a vigorous oxygen supply and to lower the temperature to 28° C. In these circumstances the addition of adrenaline to the bath in

a concentration from 1 : 12 million to 1 : 150,000 causes augmentation of the rate and force of contraction; the variation in the suitable dose of adrenaline is remarkable. It was surprising to find that butyn and stovaine both have the same action as adrenaline; thus in Fig. 4, the addition of 0.5 mg. butyn (1 : 120,000), caused a striking augmentation which lasted for 3 min.; an augmentation was also produced by stovaine in a concentration of 1 : 60,000. The isolated auricles are very different from the isolated intestine or uterus in requiring much more experience and patience if good results are to be obtained; it is, for example, not always easy to demonstrate the augmentor effect of adrenaline as the wide variation in concentration mentioned above already indicates. To obtain augmentor effects with butyn and stovaine is still more difficult, but, as I have found, it is possible. With percaïne, however, no augmentor effect was ever found, whatever dose was used; the only result was a diminution in strength and frequency of contractions.



Fig. 4. Contractions of isolated auricles of rabbit's heart suspended in Locke's solution at 28° C. The addition of 0.5 mg. butyn caused an increase of amplitude lasting for 3 min.

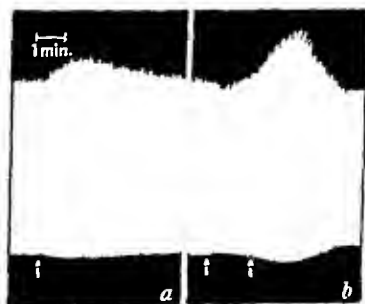


Fig. 5. Isolated rabbit's auricles, showing (a) the effect of adding 0.005 mg. adrenaline to a bath of 60 c.c., and (b) the much greater effect of the same dose of adrenaline (second arrow) after 0.1 mg. stovaine (first arrow).

Butyn and stovaine also augmented the action of adrenaline on the auricles; in Fig. 5, for example, the addition of stovaine in a concentration of 1 : 600,000, augmented the action of 5 μ g. adrenaline. Percaïne, on the contrary, abolished the action of adrenaline.

Isolated heart. In experiments on the isolated heart of the cat perfused through the coronary vessels at a temperature of $34^{\circ}\text{C}.$, the substances tested were injected into the cannula tied in the aorta. Neither butyn, percaïne nor stovaine in any dose produced an augmentation of the contractions such as is seen after the injection of adrenaline. Any dose which altered the force of the beats led either to diminution or even to arrest as Frugoni [1934] has already stated.

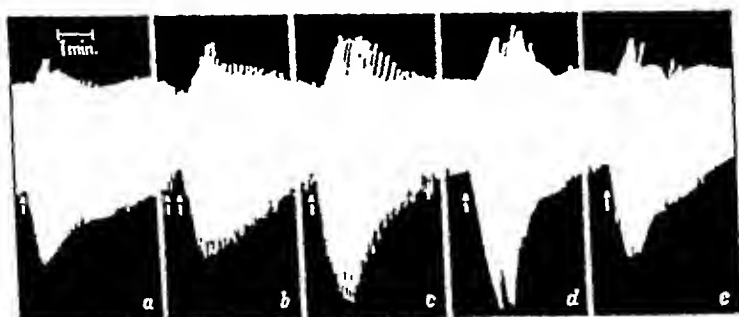


Fig. 6. Isolated cat heart, $34^{\circ}\text{C}.$, showing the increase of amplitude caused by injecting $0.5\text{ }\mu\text{g}.$ adrenaline into the cannula tied into the aorta (Langendorff). In (b), before the injection of adrenaline, $0.05\text{ mg}.$ butyn was injected as shown by the first arrow. The augmentation is clearly seen in (c) which was 1 min. later and in (d) which was $2\frac{1}{2}\text{ min}.$ later; it had disappeared in (e) 4 min. later.

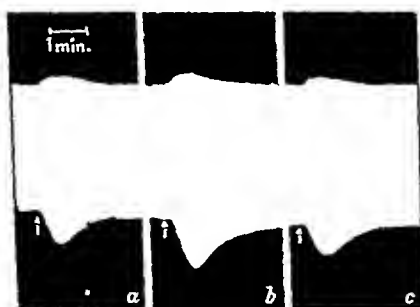


Fig. 7. Isolated cat heart. The increase in amplitude produced by injecting $0.1\text{ }\mu\text{g}.$ adrenaline into the cannula (a) before, (b) 10 sec. after, and (c) 90 sec. after the injection of $0.02\text{ mg}.$ percaïne. The injection of percaïne produced a transitory augmentation of the response to adrenaline.

On the other hand, butyn, percaïne and stovaine in small doses often influenced the response of the heart to adrenaline. For example, in Fig. 6 the injection of $0.05\text{ mg}.$ butyn temporarily increased the response

to adrenaline. A similar augmentation was found after the injection not only of stovaine, but also of percaine as shown in Fig. 7.

In certain circumstances this phase of augmentation was preceded by a brief phase of diminution, and occasionally there appeared to be diminution alone. If in different experiments the same substance in the same dose produces opposite effects, it may seem likely that neither effect is really significant. Since, however, the pressor effect of adrenaline was found to be influenced in a similar way, as is described in the next paragraph, it is preferable to accept the evidence as it stands, and to record the occurrence of both augmentation and diminution.

Pressor effects. The effect of butyn, percaine and stovaine on the blood pressure depends in part on the preparation which is used. An intravenous injection of any of these substances into the decerebrate

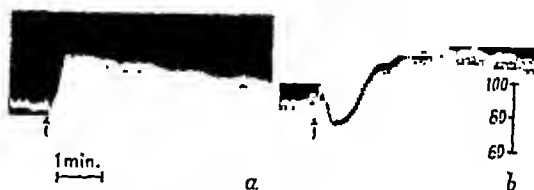


Fig. 8. To show that in occasional experiments in the spinal cat both butyn and stovaine exert a pressor action. In (a) 10 mg. butyn were injected intravenously and in (b) 10 mg. stovaine.

cat induces a fall of pressure which is transitory for butyn and stovaine, but lasts longer for percaine. In view of the toxic action on the heart which has already been mentioned it seems probable that the fall of pressure is due to weakening of the heart. When, on the other hand, the spinal preparation is used, prepared by Dale's method of dividing the spinal cord at the second cervical vertebra, injection of butyn or stovaine is sometimes followed by a rise of blood pressure. Thus Fig. 8 shows a rise of pressure produced (a) by the injection of 10 mg. butyn and (b) by 10 mg. stovaine; in (b) the rise was preceded by an initial fall. Such direct pressor effects were rare, occurring perhaps once in eight or nine experiments, the usual response being depressor as observed by Zwintz [1906] and Kamenzove [1911] in anaesthetized rabbits after the injection of stovaine and other local anaesthetics. After the injection of percaine, the blood pressure of the spinal cat fell almost to zero.

The effects of butyn and stovaine on the pressor effect of adrenaline and tyramine were of two kinds. In some experiments the pressor effect

was increased, in others it was decreased; whatever the effect on adrenaline, the same effect was observed on tyramine. In making the experiment the pressor effect of a chosen dose of adrenaline, usually 5-20 μ g. was recorded about twelve times. Butyn in a dose of 10 mg. was then injected, and at stated intervals after, the response to the same dose of adrenaline was recorded alongside one of the previous tracings. One result is shown in Fig. 9, which shows in (a) that 3 min. after the injection of butyn the adrenaline effect was slightly augmented, in (b) that

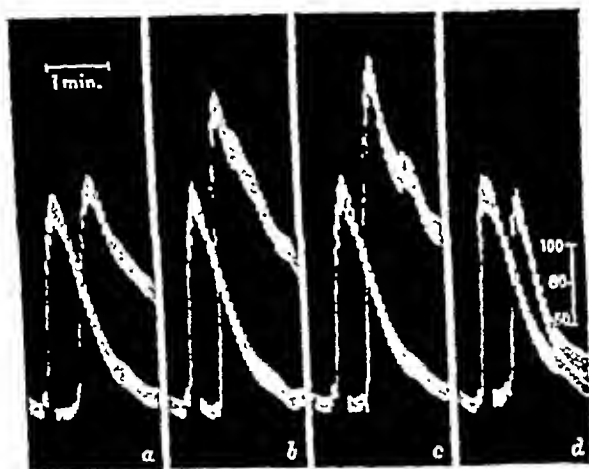


Fig. 9. Pressor effect of 0.015 mg. adrenaline (i.v.) in a spinal cat, showing the effect before and also at a certain interval after the intravenous injection of 10 mg. butyn. Thus the second injection in (a) shows the slight augmentation already present 3 min. after the butyn injection; that in (b) shows the conspicuous augmentation present 12 min. and that in (c) 40 min. after the injection of butyn. (d) shows that at 100 min. interval the pressor effect of adrenaline is the same as before the injection of butyn.

after 12 min. the adrenaline effect was greatly augmented. This augmentation reached a maximum after 40 min. as shown in (c), but disappeared after 100 min. as shown in (d). It was observed that stovaine was able to produce a similar augmentation.

Experiments on the pressor effect of tyramine were also carried out because cocaine, although it increases the pressor effect of adrenaline, diminishes that of tyramine as shown by Tainter & Chang [1927]. Fig. 10 illustrates an experiment in which stovaine was found to augment the pressor action of tyramine during the period from 20 to 40 min. after the injection of 25 mg. stovaine. Butyn was also found able to augment the effect of tyramine.

In other experiments, however, the opposite effect was seen, both after the injection of butyn and of stovaine. Fig. 11 illustrates the diminution of the adrenaline pressor effect in (a), and the diminution of the tyramine pressor effect in (b). Both results were obtained in the

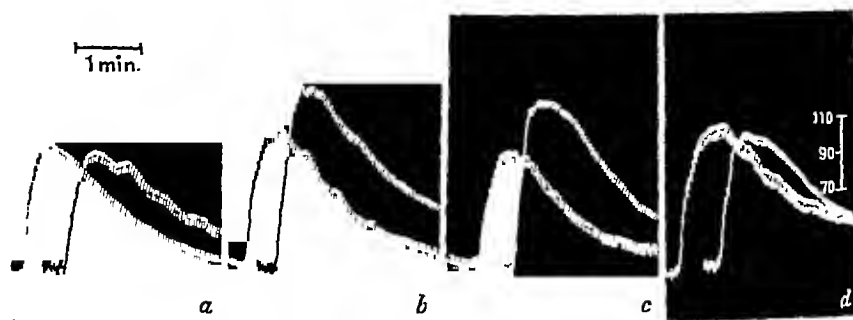


Fig. 10. Pressor effect of 1.0 mg. tyramine (base) in a spinal cat, showing the effect before and also at a certain interval after the intravenous injection of 25 mg. stovaine. The second injection in (a) was 5 min. after stovaine; that in (b) was 25 min. after; that in (c) was 40 min. after, and that in (d) was 50 min. after. (b) and (c) show a clear augmentation of the tyramine response.

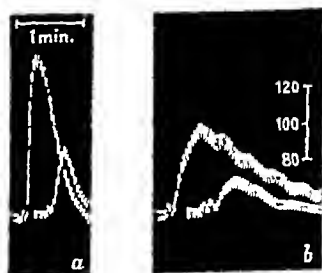


Fig. 11. This record shows that in some experiments on spinal cats the injection of butyn causes a diminution of the pressor effect of both adrenaline and tyramine. The injections in (a) were both 5 μ g. adrenaline, but the second was given 10 min. after 10 mg. butyn. The injections in (b) were both 0.1 mg. tyramine, but the second was given 7 min. after 10 mg. butyn.

same animal 7–10 min. after 10 mg. butyn, and no return of the pressor action was seen during 40 min.

With percaine the only certain result was a diminution in the adrenaline response; with small doses such as 1 mg., however, the diminution disappeared after 2 hr., and then an increased response was seen; this might have been a spontaneous change. After a larger dose, such as 5 mg., the diminution was permanent.

Effects on spleen volume. In the decerebrate cat after excluding the suprarenals from the circulation, and recording the volume of the spleen by a piston recorder, it was observed that the intravenous injection of 20 mg. butyn, 20 mg. stovaine or of 5 mg. percaine caused a diminution of the volume of the spleen similar to that caused by 1–2 μ g. adrenaline. Since, however, the diminution was accompanied by a fall of blood pressure it was impossible to know whether the change in volume of the spleen was caused by a direct constriction of the spleen muscle or was secondary to the fall in blood pressure. There was no doubt, however, about the change produced in the response of the spleen to adrenaline;

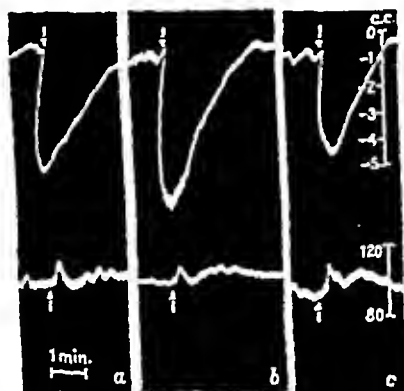


Fig. 12. Decerebrate cat with suprarenal glands excluded from circulation. Upper record is volume of spleen, and lower record is blood pressure. (a) Shows the contraction of the spleen caused by 2 μ g. adrenaline; (b) shows the augmentation of the contraction 12 min. after the intravenous injection of 5 mg. percaine; (c) shows the disappearance of this augmentation after 45 min.

Fig. 12 illustrates the augmentation of this response after the injection of 5 mg. percaine, and the disappearance of this augmentation after 45 min. A similar augmentation was observed after 20 mg. butyn and after 20 mg. stovaine.

Effects on the nictitating membrane. The observations on the cat's nictitating membrane made by Bacq [1934] have already been mentioned. I have carried out experiments on spinal cats, and have observed an alteration of the adrenaline response after the injection of percaine and stovaine. In Fig. 13 (a) is shown the contraction of the nictitating membrane caused by 6 μ g. adrenaline injected intravenously. 5 mg. percaine was then injected intravenously. In Fig. 13 (b), 5 min. later, the same dose of adrenaline caused a diminished response; in (c), 15 min.

later, the response was augmented and in (d), 20 min. later, the response had returned to the original size. When stovaine was used instead of percaïne, the adrenaline response was augmented without a phase of diminution. When butyn was injected the adrenaline response was not augmented, but appeared to be prolonged.

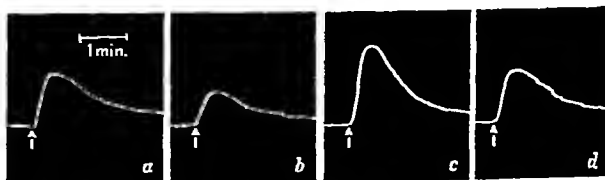


Fig. 13. Nictitating membrane of spinal cat, showing in (a) the contraction in response to the injection of 6 μ g. of adrenaline; 5 mg. percaïne was then injected intravenously; after 5 min. (b) the adrenaline response was diminished; after 15 min. (c) the response was augmented; after 20 min. (d) the original response returned.

DISCUSSION

The evidence put forward can be explained by the hypothesis that the three local anaesthetics examined all have sympathomimetic properties. Thus they have effects, some of which are identical with the effects of adrenaline and related compounds; they exert an inhibitory action on the isolated intestine of the rabbit and on the isolated uterus of the virgin cat; two of them, butyn and stovaine, augment the amplitude of the isolated auricles of the heart of the rabbit, and occasionally produce a pressor effect in the spinal cat. From the theory of the action of ephedrine put forward by Gaddum [1938], substances with a sympathomimetic action may be expected to augment the action of adrenaline by temporary combination with the enzyme which destroys it. In accordance with this it has been found that butyn and stovaine augment the action of adrenaline on the isolated auricles. All three substances augment the constrictor effect of adrenaline on the spleen, and stovaine produces a simple augmentation of the action of adrenaline on the nictitating membrane of the spinal cat. A further aspect of Gaddum's theory is the explanation of the inhibition of some adrenaline actions by ephedrine; according to this ephedrine sometimes attaches itself to sympathetic receptors and prevents the access of adrenaline. Butyn, percaïne and stovaine all diminish the action of adrenaline on the isolated uterus, this action being restored after washing.

On the blood pressure of the spinal cat and on the isolated heart of the cat butyn and stovaine affect the action of adrenaline in both ways;

sometimes they temporarily augment the adrenaline action and sometimes they depress it. We can suppose that when the effect is augmented, the combination with the enzyme is the principal result of injecting the anaesthetic, whereas when the effect is depressed the blockage of the sympathetic receptors in the cell is predominant. That a similar interpretation of the action of percaine is possible follows especially from the observations on the nictitating membrane, in which organ percaine initially depressed and then augmented the adrenaline effect. The action of percaine on the pressor effect of adrenaline appeared to be similar, in that it was initially depressed and later augmented. Thus the explanation put forward for ephedrine can be used to explain all the observations which have been made.

Earlier workers have advanced opinions concerning the action of local anaesthetics which have been diametrically opposed; some have thought they were antagonists of adrenaline, and others synergists. The former conception was upheld by Rentz & Amsler [1928] and by Fromherz [1930] who observed that the vessels of the frog perfused with a local anaesthetic no longer constricted in response to adrenaline, but dilated. The same view was taken by Laewen [1904], Meyer [1908], Günther [1916], Munch & Deckert [1930], Wirt & Tainter [1932] and more recently by Møller [1937]. The alternative conception was based on the original observation of Limbourg [1892] that the mydriatic action of cocaine was lost after degeneration of the sympathetic fibres; it was supported by the finding of Fröhlich & Loewi [1910] that cocaine augmented the pressor and the mydriatic effect of adrenaline. Further evidence in favour of a synergism between adrenaline and local anaesthetics came from the work of Hatcher & Eggleston, who [1916] noted that novocaine (procaine) increased the pressor action of adrenaline, and [1919] found a similar effect to be produced by other local anaesthetics. In 1939 Crosby made observations on the vessels in the rabbit's cornea from which he concluded that local anaesthetics, especially cocaine, augmented the constrictor action of adrenaline.

By the adoption for local anaesthetics of Gaddum's theory of the action of ephedrine, the two views of antagonism and synergism can now be harmonized. Naturally, there remain various obscurities which are not satisfactorily explained; for example, the conditions in which butyn or stovaine will augment the pressor action of adrenaline or in which they will depress it are not yet defined; similarly, in any given experiment on the isolated heart it is impossible to foretell whether butyn or stovaine will augment or depress the action of adrenaline. Finally, it is not known

later, the response was augmented and in (d), 20 min. later, the response had returned to the original size. When stovaine was used instead of percaine, the adrenaline response was augmented without a phase of diminution. When butyn was injected the adrenaline response was not augmented, but appeared to be prolonged.

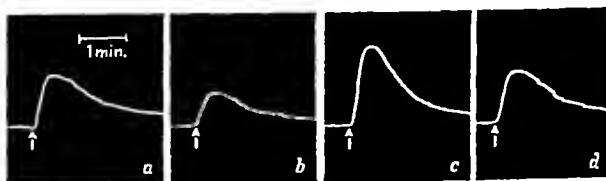


Fig. 13. Nictitating membrane of spinal cat, showing in (a) the contraction in response to the injection of 6 μ g. of adrenaline; 5 mg. percaine was then injected intravenously; after 5 min. (b) the adrenaline response was diminished; after 15 min. (c) the response was augmented; after 20 min. (d) the original response returned.

DISCUSSION

The evidence put forward can be explained by the hypothesis that the three local anaesthetics examined all have sympathomimetic properties. Thus they have effects, some of which are identical with the effects of adrenaline and related compounds; they exert an inhibitory action on the isolated intestine of the rabbit and on the isolated uterus of the virgin cat; two of them, butyn and stovaine, augment the amplitude of the isolated auricles of the heart of the rabbit, and occasionally produce a pressor effect in the spinal cat. From the theory of the action of ephedrine put forward by Gaddum [1938], substances with a sympathomimetic action may be expected to augment the action of adrenaline by temporary combination with the enzyme which destroys it. In accordance with this it has been found that butyn and stovaine augment the action of adrenaline on the isolated auricles. All three substances augment the constrictor effect of adrenaline on the spleen, and stovaine produces a simple augmentation of the action of adrenaline on the nictitating membrane of the spinal cat. A further aspect of Gaddum's theory is the explanation of the inhibition of some adrenaline actions by ephedrine; according to this ephedrine sometimes attaches itself to sympathetic receptors and prevents the access of adrenaline. Butyn, percaine and stovaine all diminish the action of adrenaline on the isolated uterus, this action being restored after washing.

On the blood pressure of the spinal cat and on the isolated heart of the cat butyn and stovaine affect the action of adrenaline in both ways;

THE INHIBITION OF ADRENALINE OXIDATION BY LOCAL ANAESTHETICS

By FLORA J. PHILPOT

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(Received 7 July 1939)

It has been suggested on several occasions that substances which potentiate the action of adrenaline might do so by inhibiting its removal or destruction (a case analogous to that of eserine and acetylcholine [cf. Bain, Gaunt & Suffolk, 1937; Bayer & Wense, 1938]). Blaschko, Richter & Schlossmann [1937] showed that ephedrine inhibits the amine oxidase which oxidizes the side-chain of adrenaline. Other related amines also inhibit the oxidation of adrenaline by competing with it for adsorption on the enzyme, e.g. tyramine, hordenine, corbasil. Gaddum & Kwiatkowski [1938] showed that ephedrine sensitized the rabbit's ear, the cat's nictitating membrane and the frog's heart not only to adrenaline but also to the stimulation of adrenergic nerves. They suggest that the potentiating action of ephedrine is due to inhibition of the amine oxidase.

Bain *et al.* [1937] found that the addition of cocaine did not affect the rate of destruction of adrenaline incubated with liver slices, but they used extremely low concentrations of adrenaline and cocaine. Bayer & Wense [1938] and Wense [1939] claim that cocaine slows the rate of oxidation of adrenaline by tyrosinase, acetaldehyde and succinic acid. In the last two cases a peroxide is formed which oxidizes the adrenaline to adrenochrome.

Recently, Macgregor [1939] has extended Gaddum's hypothesis to the action of cocaine and procaine; and Tripod [1940] has continued this work to include three other local anaesthetics, stovaine, butyn and percaine. All these substances have sympathomimetic properties which can be explained along the lines of Gaddum's hypothesis. It was therefore decided to study the action of these five local anaesthetics on the enzymic oxidation of adrenaline.

whether the sympathomimetic action of local anaesthetics has any quantitative relation to their local anaesthetic power, though in the experiments described, percaine, which is by far the most powerful anaesthetic, was used for sympathomimetic effect in much the lowest dose.

SUMMARY

The local anaesthetics, butyn, percaine and stovaine, have the following direct sympathomimetic effects:

- (1) They inhibit the isolated intestine of the rabbit.
- (2) They inhibit the isolated uterus of the cat.
- (3) They augment the contractions of the isolated auricles of the rabbit.

In addition they modify the action of adrenaline, either augmenting or depressing it. Thus they augment or depress the action of adrenaline

- (1) on the isolated heart of the cat;
- (2) on the blood pressure of the spinal cat;
- (3) on the nictitating membrane of the cat;
- (4) on the spleen volume of the cat.

All these effects can be regarded as sympathomimetic and explained by substrate competition for the molecules of enzyme, or by receptor competition.

The work described above has been carried out with the help of a scholarship from the Cesar Roux Foundation of the University of Lausanne. The work has been done under the direction of Prof. J. H. Burn to whom I wish to express my thanks.

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TABLE II. Inhibition of adrenaline oxidation by amine oxidase

Inhibitor	Molarity	Adrenaline molarity	% inhibition
Ephedrine	0.015	0.0055	50
Procaine	0.0175	0.0055	60
Butyn	0.015	0.0055	75
Stovaine	0.015	0.0055	81
Cocaine	0.0175	0.011	80
Percaine	0.0075	0.0055	90

The following volumes were used: enzyme, 0.5 ml.; adrenaline, 0.2 ml.; phosphate buffer, 0.7 ml.; local anaesthetic, 0.3 ml.; semicarbazide, 0.1 ml.; KCN, 0.1 ml.; KOH, 0.1 ml.; final volume, 2.0 ml. The rate was measured as the extra oxygen uptake, over that of the control, during the period 0-20 min.

The fact that the concentrations used are not identical for all the compounds tested makes an exact estimate of their potencies difficult. It was evident, however, in the course of the work that ephedrine and procaine were considerably less potent, and percaine more potent than cocaine, stovaine and butyn. No attempt has yet been made to estimate the relative potencies *in vivo*, but, judging from the doses used by Tripod and Maegregor, procaine is weaker than butyn and stovaine, and these in turn are weaker than percaine. Ephedrine has not been used in experiments which can be compared with these.

Oxidation to adrenochrome

Green & Richter [1937] found that adrenaline is rapidly oxidized by heart muscle to the ortho-quinone. Ring closure by the side-chain follows, giving a red substance, adrenochrome, which acts as a respiratory carrier, similarly to methylene blue. This substance they found was without action on the blood pressure of the spinal cat. It was decided to test it in a variety of ways to see whether adrenochrome had any physiological action to correspond with its apparent biochemical one.

Adrenochrome was prepared by incubating adrenaline with a 20% pure preparation of catechol oxidase from mushroom (kindly given to me by Prof. D. Keilin) at pH 5.0. The oxygen uptake was followed in a manometer and the incubation ended when the oxygen uptake had almost ceased. According to calculations, based on the oxygen uptake, the conversion of adrenaline to adrenochrome should have been about 95%. This solution was tested on the perfused cat's heart, the virgin cat's uterus, the nictitating membrane and blood pressure of a spinal cat. In all cases it was diluted at the last minute before use and the stock solution was kept at 0° C. The effects produced were matched against varying doses of adrenaline. The conclusion from the experiments was that the

TECHNIQUE

Rat or guinea-pig liver was used as a source of the amine oxidase. The liver was frozen solid under Ringer's solution, ground in a mortar and squeezed through muslin. The pulp was centrifuged (3000 rev./min. for 20 min.) and the sediment was suspended in phosphate buffer 0.1M, pH 7.0. This procedure was sufficient to reduce the residual oxygen uptake to very low values.

The oxygen uptake was measured by the Warburg manometric technique, in air, at 38°. The substrate was added from the side-tube after 10 min. temperature equilibration. 2N KOH was used to absorb CO₂. Experiments were done with tyramine and adrenaline as substrates. When adrenaline was used, KCN and semicarbazide were added, the former to prevent oxidation of adrenaline by other enzyme systems, the latter to remove the oxidation product, which is an aldehyde [cf. Blaschko *et al.* 1937]. The following solutions were used:

Acetate buffer 0.1M, pH 5.0; phosphate buffer 1.0M, pH 7.0; tyramine 0.14M; adrenaline HCl 0.11–0.055M; KCN 0.03M; semicarbazide 1.0M; the following hydrochlorides were 0.1M, cocaine, procaine, stovaine, butyn, percaïne, and ephedrine; cytochrome C 2.65 × 10⁻⁴M; *p*-phenylene-diamine HCl 0.2M.

EXPERIMENTAL RESULTS

Oxidation in the side-chain by amine oxidase

Tyramine. The rate of oxidation of tyramine by liver suspensions is inhibited by both cocaine and procaine. The degree of inhibition depends on the relative concentrations of inhibitor and substrate. Table I shows typical figures obtained with rat and guinea-pig livers.

TABLE I. Inhibition of tyramine oxidation by amine oxidase

Tyramine M	0.0048	0.0048	0.0048	0.0048	0.0097	0.0143
Procaine M	0.0334	—	—	—	—	—
Cocaine M	—	0.0033	0.01	0.0167	0.0167	0.0167
% inhibition	75	26	40	65	40	30

The following volumes were used: enzyme, 0.5 ml.; tyramine, 0.1 ml.; phosphate buffer, 0.7 ml.; cocaine and procaine, 0.3 ml.; KOH, 0.1 ml.; final volume, 2.0 ml. The rate was measured as the extra oxygen uptake, over that of the control, during the period 0–20 min.

Adrenaline. The oxidation of adrenaline by rat liver was sometimes difficult to demonstrate as it was very slow. Guinea-pig liver gave a good rate of oxidation. All the anaesthetics tested produced in both cases a marked inhibition in the rate of oxidation, as shown in Table II. Ephedrine was tested to give a comparison.

TABLE II. Inhibition of adrenaline oxidation by amine oxidase

Inhibitor	Molarity	Adrenaline molarity	% inhibition
Ephedrine	0.015	0.0035	50
Procaine	0.0175	0.0035	60
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Percaine	0.0075	0.0035	90

The following volumes were used: enzyme, 0.5 ml.; adrenaline, 0.2 ml.; phosphate buffer, 0.7 ml.; local anaesthetic, 0.3 ml.; semicarbazide, 0.1 ml.; KCN, 0.1 ml.; KOH, 0.1 ml.; final volume, 2.0 ml. The rate was measured as the extra oxygen uptake, over that of the control, during the period 0-20 min.

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small amount of activity found in all four tests was due to a remnant of unconverted adrenaline; this activity diminished during the time in which the observations were actually made. For example, in one experiment the first observations were made on the isolated heart and they indicated that in the adrenochrome solution there was activity corresponding to 6% of the adrenaline originally present. The next observations were made on the blood pressure and nictitating membrane of the spinal cat; they showed activity corresponding to not more than 3% of the adrenaline originally present. At first it was thought that this difference was to be explained by the difference between the potency of adrenochrome in relation to that of adrenaline on the isolated heart, on the one hand, and on the blood pressure on the other. Further tests on the isolated heart were then made, which showed that the activity corresponded at that stage to no more than 1% of the adrenaline originally present. Since the solution still contained the enzyme it seemed probable that all the activity was due to residual adrenaline which was slowly undergoing oxidation. Control tests were made which showed that the presence of the enzyme in the adrenochrome solution did not affect the results.

Since adrenochrome itself has no physiological action it is clear that the normal path of oxidation of adrenaline might be via the orthoquinone. The action of the local anaesthetics and ephedrine was therefore tested on catechol oxidase, both on a crude preparation and on the 20% pure preparation, and on the cytochrome-cytochrome oxidase system which also oxidizes adrenaline to adrenochrome [Green & Richter, 1937]. Cytochrome oxidase was prepared according to Ogston & Green [1935]. The cytochrome was made by Mr Stocken in the Department of Biochemistry.

Ephedrine, procaine, cocaine and percaïne inhibited the rate of oxidation by both enzymes by 25% or less, even with a ratio of inhibitor/substrate which would give 100% inhibition with the amine

TABLE III. Inhibition of adrenaline and *p*-phenylene-diamine oxidation by cytochrome oxidase

Inhibitor	Molarity	Adrenaline molarity	<i>p</i> - ϕ -diamine <i>M</i>	% inhibition
Cocaine	0.015	0.00275	—	21
"	0.015	—	0.02	22
Procaine	0.015	0.00275	—	25
"	0.015	—	0.02	10
Ephedrine	0.015	0.00275	—	15
"	0.015	—	0.02	10

Cytochrome *C* was present in all experiments in a final concentration of $0.133 \times 10^{-4} M$. Other details as in Table I.

oxidase. Table III shows the results with adrenaline and *p*-phenylenediamine as reducing agents in the cytochrome oxidase system. Table IV shows the results with adrenaline as substrate for catechol oxidase. It is noteworthy that ephedrine, procaine, cocaine and percarine are all equally efficient as inhibitors for these two enzymes.

TABLE IV. Inhibition of adrenaline oxidation by catechol oxidase

Inhibitor	Molarity	Adrenaline molarity	% inhibition
Cocaine	0.0175	0.00275	25
Procaine	0.015	0.00275	25
"	0.0075	0.00275	20
Ephedrine	0.0175	0.00275	25

The following volumes were used: enzyme, 0.5 ml.; adrenaline, 0.1 ml.; acetate buffer, 1.0 ml.; anaesthetic, 0.35 ml. The rate was measured as the oxygen uptake during the period 0-15 min.

Other enzymes

To find out whether the local anaesthetics were general enzyme inhibitors cocaine and procaine were tested on the xanthine oxidase of milk and the malic and succinic dehydrogenases of heart muscle. No inhibition was observed.

DISCUSSION

The finding of Bayer & Wense that cocaine inhibits the oxidation of adrenaline by peracetic acid (from acetaldehyde) does allow the possibility that the normal destruction of adrenaline is non-enzymic and, as they suggest, connected with the production of aldehydes (and hence peroxides) during carbohydrate metabolism. There is no evidence that acetaldehyde is formed during carbohydrate metabolism, and, if it were, it would be rapidly converted by mutases to the corresponding acid and alcohol. Hydrogen peroxide itself does not act on adrenaline at pH 7.0 in absence of a catalyst. It seems reasonable, therefore, to assume that the normal destruction of adrenaline is enzymic.

As we have seen, there are two possible ways in which enzymic oxidation may occur. The support for the view that oxidation occurs in the benzene ring to yield adrenochrome comes from the following considerations.

(1) The cytochrome-cytochrome oxidase system is very widely distributed, whereas the amine oxidase has only been demonstrated in liver, kidney, brain and intestine. Similarly polyphenol oxidases have a wide distribution.

(2) Bacq [1938] has suggested that the inhibitory action of adrenaline on the virgin cat's uterus and on intestine is due to the formation of

adrenoxine (an oxidation product of adrenochrome), which has an action differing from that of adrenaline itself.

Against the first point we have the evidence that the local anaesthetics and ephedrine inhibit cytochrome oxidase and catechol oxidase to a relatively small extent compared with their action on the amine oxidase. Similarly there is no apparent parallel between the potency of the different inhibitors on the former enzymes *in vitro* and their potency *in vivo*. Secondly if Bacq's suggestion were true adrenochrome should inhibit the virgin cat's uterus as efficiently as does adrenaline. Actually adrenochrome was found to have no action, other than that due to the remnant of unconverted adrenaline.

Objections can also be raised to the theory that the amine oxidase is responsible for the *in vivo* oxidation of adrenaline, but they are not unanswerable.

(1) It is clear from experimental work that some mechanism exists for removal of adrenaline *in situ*, e.g. in the perfused rabbit's ear. Up to the present there has been no attempt to demonstrate amine oxidase activity in such an organ. But with the newly available micro-respirometry techniques [Heatley, Berenblum & Chain, 1939] it should be possible to investigate the enzymes on the spot. Until that is done there is no evidence against the supposition that the amine oxidase may be very widely distributed, though present in amounts too small to demonstrate by the ordinary methods.

(2) Kohn [1937] dismisses amine oxidase as being of no physiological importance because of the low affinity of the enzyme for adrenaline. This argument cannot be regarded as final in view of the fact that we are concerned with an insoluble enzyme. The conditions in *in vitro* experiments are likely to be far less favourable to the enzyme than when it is correctly orientated in the cell, so there can be no prediction of the actual *in vivo* affinity of the enzyme.

(3) The conclusions of Bain *et al.* that cocaine does not inhibit the destruction of adrenaline can be criticized on several grounds. Since the inhibition by cocaine is competitive it follows that the degree of saturation of the enzyme and the concentration ratio inhibitor/adrenaline are important factors in determining the amount of inhibition. Under their conditions the enzyme was far from saturated and the cocaine/adrenaline ratio was 0.5. In the experiments described here the enzyme was saturated and the ratio was between 3 and 6; and in Gaddum's experiments with ephedrine on the rabbit's ear the ratio was 10. If the theory of the action of local anaesthetics on the amine oxidase is to hold we must

postulate a high degree of saturation of the enzyme *in vivo*. This could only occur if the enzyme were located at the site of action of adrenaline and was readily saturated by small amounts of adrenaline.

Owing to the great difference in the conditions obtaining between *in vivo* and *in vitro* experiments, and the much higher concentrations usually required to produce measurable effects in the latter, great caution must be observed when arguing from one to the other. This applies especially to quantitative comparisons.

Thus, although the evidence appears to point to amine oxidase as the enzyme responsible for oxidizing adrenaline *in vivo*, it would be unwise to dismiss entirely the adrenochrome route in favour of the amine oxidase route merely on the evidence produced here; i.e. that the inhibition of the latter *in vitro* is more potent; and that there is a parallel between the potencies of the local anaesthetics *in vitro* and *in vivo* where the amine oxidase is concerned, but no such parallel where the oxidation to adrenochrome is concerned.

SUMMARY

1. Cocaine, procaine, stovaine, butyn and percaïne inhibit the amine oxidase of rat and guinea-pig liver by 60-100%.
2. In the same concentrations cocaine, procaine, percaïne and ephedrine inhibit cytochrome oxidase and catechol oxidase by 10-25%.
3. Cocaine and procaine do not inhibit xanthine oxidase or the malic and succinic dehydrogenases of heart muscle.
4. Adrenochrome has no physiological activity on the blood pressure of the spinal cat, the nictitating membrane, virgin cat's uterus or isolated cat's heart.
5. The destruction of adrenaline *in vivo* and the potentiating actions of local anaesthetics and ephedrine are discussed in view of the above results.

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ON THE PRIMARY ACIDITY OF THE GASTRIC JUICE

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As early as 1898 Pavlov advanced the theory that the gastric hydrochloric acid is secreted from the mucosa glands at a *high and constant concentration*, independent of other conditions such as secretion rate, etc. This so-called "primary acidity" undergoes secondarily a reduction to lower and variable values, owing to interference from various acidity regulating factors.

Most workers on gastric physiology have adopted Pavlov's view, although so far nobody has been able to examine the primary juice directly. There are many, however, who regard the hydrochloric acid concentration of the "primary juice" as being variable according to the degree of stimulation of the stomach cells [Roseman, 1927; Katsch & Kalk, 1926; and others]. Hollander [1932], in particular, has defended the Pavlov thesis and has attempted to accumulate *indirect* evidence in its favour. He claims that the primary acidity corresponds to blood isotonicity (*ca.* 167 mN.¹). His evidence is based on inferences from the observed correlation between acidity and neutral chloride of gastric juice samples. Hollander's method of arguing is purely mathematical and involves an extrapolation to the acidity value where the neutral chloride is *zero*. The fact that this extrapolation leads to a constant figure of about 167 mN. does not necessarily prove that the primary acidity of samples containing neutral chlorides is also 167. A similar mathematical procedure employed by Liu, Yuan & Lim [1934] is open to the same criticism. Although the Pavlov-Hollander hypothesis is attractive in many respects, from a critical standpoint one must conclude that it lacks a convincing foundation.

The object of the present study was to attempt a direct examination of the acidity of the primary juice. The mode of attack was based on new concepts as regards the mechanism of the acidity regulation [the

¹ mN. = milli-normal = milli-equivalents per litre.

"diffusion theory", Teorell, 1933, 1935, 1939a; cf. also Engeström, 1935; Ihre, 1938].

*Remarks on the acidity regulation.*¹ Regardless of their views on the composition of the primary juice, all workers in this field agree that there exist secondary acidity-reducing factors. The nature of these factors is still a matter of controversy. Pavlov [1898] and many later investigators consider a neutralization by gastric mucus as the most important factor. Others have suggested a neutralization process by bicarbonate or buffers of extra-gastric origin (Boldyreff's [1911] duodenal regurgitation), or of intragastric origin [Hollander, 1938]. There are many who believe in a pure dilution by an intragastric "Verdünnungsssekretion" [McLean, 1928; Katsch and Kalk, 1926; and others]. Working with gastric juice secretion of cats, induced by histamine stimulation, the writer [1933] was unable to find indications of neutralizing or diluting processes. Instead he was forced to adopt a purely physico-chemical view and advanced a "diffusion theory" as the only satisfactory explanation of the experimental findings. The chief principle is the following: the hydrochloric acid diffuses steadily out of the stomach content into the mucosa cells or the blood, and is exchanged against alkali chlorides ("neutral chlorides") which diffuse the other way. Expressed technically, there is a continuous process of ionic interdiffusion or exchange diffusion going on in the stomach. (For details of this theory see Teorell [1933, 1939a].)

Principle of experimental procedure. Experiments on the introduction of acids into cats' stomachs show that *the loss of acidity is proportional to the prevailing hydrogen-ion concentration* [Teorell, 1933, 1939 a], in accordance with the laws of diffusion and the behaviour in model experiments [Teorell, 1939 b]. From this it follows that there are two ways open for a prevention of secondary acidity reduction:

(a) A lowering of the hydrogen-ion concentration of the stomach content by *dilution* of the acid secretion. This method has been utilized by Engeström, who received the HCl secretion in a large volume of water introduced into the stomach. A drawback, however, is that very large volumes of diluting solutions have to be employed in order to prevent HCl loss completely (for example, a reduction of the back diffusion rate to a tenth requires a diluting volume which is nine times larger than that of the acid secretion).

(b) A lowering of the hydrogen-ion concentration by mixing the acid juice with a suitable *buffer* solution. Provided this has a sufficiently large acid-binding capacity, it is possible to use comparatively small volumes of the buffer.

In either case one can determine the *amount* of HCl secreted by titrating the buffer and juice withdrawn with NaOH back to the pH of the original juice-free buffer. If the solution introduced is osmotically

¹ The subject of primary acidity and acidity regulation has recently been reviewed by Hollander [1938]. Cf. also the dissertations by Ihre [1938], Engeström [1935] and Teorell [1933].

so adjusted that it does not undergo any volume changes in the stomach, it is obvious that any increase of volume is a direct measure of the *volume* of the secretion. Finally, by dividing the amount by the volume one obtains the *concentration* of HCl in the secretion. The following numerical example illustrates the principle:

5.0 c.c. of an "isotonic" buffer (pH 5.9) were introduced into a just emptied but secreting stomach. After a certain time 6.5 c.c. could be withdrawn from the stomach. [The pH was now 2.75. Without buffer the pH would probably have been about 1.0, hence there has been achieved a 56-fold reduction of the acidity ($2.75 - 1.0 =$ the logarithm of 56).] The sample withdrawn required 2.8 c.c. $N/10$ NaOH for adjustment of the pH back to 5.9, i.e. the amount of HCl secreted corresponds to 0.28 mM. The volume increment was $6.5 - 5.0 = 1.5$ c.c., hence the *primary acidity* of the secretion is equal to $\frac{0.28 \times 1000}{1.5} = 186 \text{ mN}$.

METHODS

After several preliminary trials a suitable buffer substance was found in glycol. In a 0.2–0.3 M solution it is subject to insignificant volume changes in the stomach. The substance is very poorly permeable through the mucosa (cf. Teorell [1939 *a*], and the total nitrogen column of Table II), therefore, its buffering capacity remains practically constant. The poor permeability probably explains why one has to employ solutions hypotonic to the blood in order to avoid osmotic volume changes (a 0.2 M solution has a freezing point of 0.37° compared with 0.60° of the cat's blood). At 6.0, the pH of the plain glycol solution, the buffering capacity is quite small, therefore a sharp end-point for the back titration is obtained (see Exp. 1 (*b*)).

The pH measurements and NaOH titrations were performed by aid of a glass electrode arrangement. The chloride titrations were carried out electrometrically. Nitrogen determinations were made on aliquot parts, as described by Teorell [1928].

The volumes were measured directly, the accuracy being ± 0.2 c.c.

The treatment of the experimental animals was the same as described in detail elsewhere [Teorell, 1939 *a*].

The mode of calculation is illustrated above and by Exps. 1 and 2.

RESULTS

Prior to the main experiments some controls were performed in order to test the reliability of the methods employed. The results are quoted as Exps. 1–4.

Experiment 1. Titration controls in vitro.

(a) 5.0 c.c. of a 0.9% NaCl + 2.0 c.c. of 0.177 *N* HCl were titrated with *N*/10 NaOH. End-point pH 7.0, total NaOH consumption 3.54 c.c. Volume "increment" 2.0 c.c., hence the "primary acidity" is equal to $\frac{0.354}{2.0} \times 1000 = 177$ mN. (calc. 177).

(b) 5.0 c.c. of 0.2 *M* glycerol + 2.0 c.c. of a 0.177 *N* HCl titrated with *N*/10 NaOH. End-point pH = 6.0.

c.c. NaOH	...	Initial	1.20	2.00	2.50	3.00	3.30	3.50	3.60	
pH	2.60	2.90	3.13	3.32	3.66	4.12	5.86	7.75

NaOH consumption 3.55 c.c. Volume "increment" 2.0 c.c., hence the "primary" acidity is equal to $\frac{0.355}{2.0} \times 1000 = 178$ mN. (calc. 177).

N/10 AgNO₃ consumption 3.56 c.c. (calc. 3.54).

Exp. 2. Recovery controls in cat's stomach. Glycerol + HCl mixtures (a slight fasting secretion was going on).

(a) 5.0 c.c. of a solution (5.0 c.c. 0.2 *M* glycerol + 2.0 c.c. 0.177 *N* HCl) were introduced into the emptied stomach (at 12.20 p.m.). At 12.35 a volume of 5.4 c.c. was recovered (pH 2.54) which consumed 3.40 c.c. *N*/10 NaOH (end-point pH 5.5-6). The *N*/10 AgNO₃ consumption of the total sample was 4.20 c.c.

The theoretical NaOH consumption corresponding to the amount of HCl introduced ought to be $\frac{5}{1} \times \frac{2 \times 0.177}{0.1} = 2.53$ c.c. This figure is lower than the figure actually determined, indicating the presence of an acid secretion corresponding to $3.40 - 2.53 = 0.87$ c.c. *N*/10 NaOH. If one postulates the primary acidity to be 0.175 *N*, the secreted volume is calculated to be $(0.1 \times 0.87) - 0.175 = 0.50$ c.c. Now, the actual volume increment was $5.4 - 5.0 = 0.4$ c.c., hence there is a deficit of 0.1 c.c.

If it is assumed that all hydrogen ions were secreted together with chloride ions, one can calculate the "neutral" chloride concentration of the stomach content to be $\frac{(4.20 - 3.40)}{5.4} \times 100 = 15$ mN.

(b) The foregoing procedure was repeated: 5.0 c.c. fresh glycerol + HCl mixture were introduced at 12.35 p.m., 5.0 c.c. were recovered at 12.50 (pH 3.48). NaOH consumption 2.95 c.c., AgNO₃ consumption 3.57 c.c.

Acid increment corresponding to $2.95 - 2.53 = 0.42$ c.c. NaOH or 0.24 c.c. primary acid. The actual increment was 0, hence a volume deficit of 0.24 c.c.

The neutral chloride concentration was $\frac{(3.57 - 2.95)}{5.0} \times 100 = 12$ mN.

Exp. 3. Recovery controls in cat's stomach. Glycerol alone (a slight fasting secretion was still going on).

(a) 5.0 c.c. of 0.2 *M* glycerol were introduced into the emptied stomach at 1.10 p.m., 5.0 c.c. (pH 3.52) were recovered at 1.25. NaOH consumption 0.55 c.c. corresponding to 0.31 c.c. of primary acid, hence a deficit of 0.31 c.c., AgNO₃ consumption 1.17 c.c., neutral chloride concentration equal to 12 mN.

Total nitrogen of the sample was 13.8 mg. (calc. 14.0).

(b) Foregoing repeated 1.30-1.45. 5.0 c.c. recovered (pH 3.66) NaOH consumption 0.50 c.c., AgNO₃ consumption 1.08 c.c., yielding a volume deficit of 0.29 c.c. and a neutral chloride value of 12 mN.

Total nitrogen of the sample 13.8 mg. (calc. 14.0).

From the Exps. 1-4 one can infer that:

(1) the titration methods are satisfactory; (2) there is a slight water "resorption" of 0.2-0.3 c.c. during the time interval of 15 min.; (3) there occurs an increase of the neutral chloride level up to about 12 mM. during 15 min.; (4) the glycol does not diffuse out of the stomach (N is constant).

The main experiment is recorded in Table I. When calculating the primary acidity the volume was corrected with +0.2 c.c. to account for the water resorption.

TABLE I. Exp. 4. Gastric juice secretion in the presence of glycol buffer. The same cat as in Exp. 3. Every 15 min. total emptying of stomach content and 5.0 c.c. fresh glycol solution introduced. At 1.48 p.m. subcutaneous injection of 0.6 mg. histamine hydrochloride.

hydrochloride.				Concentration					
Time	Volume re-covered	Volume in-crement	pH	Total N of sample mg.	Concentration			$H^* \div$ Total Cl	Primary acidity† mN.
					H^* mN.	Total Cl mN.	Neutral Cl mN.		
1.50	(5.0 c.c. introduced)		5.46	(14.0)‡	—	—	—	—	—
2.05	6.15	1.15	2.94	15.7	36	42	6	0.86	163
2.20	8.00	3.00	2.38	16.2	70	70	0	1.00	175
2.35	8.00	3.00	2.16	—	84	85	1	0.99	209
2.50	7.7	2.7	2.16	—	83	83	0	1.00	213
3.05	7.9	2.9	2.22	—	78	80	2	0.98	200
3.20	6.5	1.5	2.40	14.4	69	74	5	0.93	264
3.35	5.6	0.6	2.83	—	45	54	11	0.83	310
3.50	5.4	0.4	3.08	13.8	30	34	4	0.88	266
4.05	5.5	0.5	3.10	—	27	38	11	0.71	216
4.20	5.25	0.25	3.30	—	18	28	10	0.64	211
4.35	5.15	0.15	3.44	13.1	16	24	8	0.67	228

* H denotes titratable acidity to pH 5.5-6.

† 0.2 c.c. has been added to the volume increment to allow for water resorption.

‡ Calculated value.

The results of Exp. 4 (Table I) should be compared with those of Exp. 5 (Table II) which is a standard gastric juice experiment where "secondary" acidity only was determined.

TABLE II. Exp. 5. Gastric juice production without extragastric admixture. (The same cat as in Exps. 1-4. Every 15 min. total emptying of the stomach. At 8.20 p.m. subcutaneous injection of 0.6 mg. histamine hydrochloride.)

Time p.m.	Volume recovered c.c.	Total N of sample mg.	Total acidity* mN.	Total chloride mN.	Neutral chloride mN.
8.20	—	—	—	—	—
8.35	0.7	0.4	76	186	110
8.50	3.6	2.1	148	173	25
9.10	5.2	1.0	156	189	33
9.25	4.4	0.9	164	188	24
9.40	3.5	1.2	153	182	29
9.55	2.2	—	138	180	42
10.10	1.5	—	115	173	57
10.25	0.7	—	94	164	70

* End-point pH 7.4.

A comparison between Tables I and II shows the following:

(1) The *primary acidity* lies on a considerably higher level than the *total acidity*, and appears to be independent of the rate of secretion. There is a considerable fluctuation among the figures obtained, between 63–310 mN. It should be remembered, however, that these figures are very sensitive to errors in the volume determinations. A deficit within the limits of error, 0.2 c.c., causes a 10 % increase of the primary acidity when the volume increment is 2 c.c. and still more when the increments are smaller. It may therefore be justified to disregard all samples with less than 2 c.c. increments, in which case the following figures only can be accepted as reliable

175 209 213 200 mN.

A similar experiment on another cat yielded for four different samples

222 224 219 202 mN.

The average of these eight figures for the primary acidity is 208 ± 6 mN.

(2) The neutral chloride level in the glyecol experiments is somewhat fluctuating. The neutral chloride level in the standard experiment is considerably higher. The reason for this behaviour will be considered in the discussion.

(3) There is no increased loss of glyecol due to diffusion during actual gastric juice secretion. This is shown by the nitrogen figures.

DISCUSSION

The main error in the method employed rests in the difficulty of obtaining accurate volume figures. Dilution measurements with the aid of a fixed "reference substance" (phenol red, etc.) would perhaps improve the results. Owing to this circumstance one can only ascribe significance to figures obtained at the higher secretion rates.

It can also be objected that the back titration with NaOH over the pH range 2.5–6 includes not only the HCl + glyecol-HCl, but also other base binding substances which might appear in the stomach contents. It can likewise be suggested that some HCl can be neutralized by other substances than the buffer, having an acid binding power beyond pH 5.5–6. Of these two opposing possibilities the former is the most probable one, as indicated by the very small neutral chloride figures recorded in Table I. Hence it is likely that we have been dealing with NaOH titration values which are slightly too high; these in turn have given rise to primary acidity figures which are somewhat too high.

The average value of the primary acidity so obtained, 208 ± 6 mN.,

would even after due allowance for a possible "extra" base binding, remain higher than the figure 165-170, indirectly arrived at by Hollander [1932]. It would agree rather with the suggestions by Liu, Yuan & Lim [1934] that the parietal secretion is hypertonic with respect to blood. However, the total chloride values of the acid juice from cats are in general higher than in dog or human, values above 180 mM. being not uncommon (cf. Table II). Therefore, the matter of the possible isotonicity of the primary juice with respect to the blood cannot be decided upon until freezing-point determinations or appropriate calculations of the osmotic pressures have been performed.

In several communications it has been shown that there is an inward diffusion of alkali chlorides from the mucosa or blood to the stomach contents [Teorell, 1933, 1939 *a*], and it is therefore most natural to explain the excess chloride over the "acid" chloride, i.e. the neutral chloride, as a product of such an inward diffusion.

The results obtained here may allow some speculations as to the position of the mucosa elements in which the back diffusion of hydrochloric acid takes place. It is generally assumed that the parietal secretion enters the stomach cavity via the "crypts" of the mucosa glands. As the crypts are microscopically narrow, it seems likely that the outwardly directed stream of secretion prevents the buffer from entering them. As nevertheless the figure of primary acidity was found to be high and, so far as could be judged, independent of the secretion rate, it might be inferred that no acidity reduction occurred during the passage through the crypts. The evidence is not conclusive; on the contrary, there are some signs which may indicate that some "intraglandular acidity reduction" [Engeström, 1935] has occurred, at least at the lowest secretion rates. These signs are the decreasing ratios (titrable acidity/total chloride) of Table I. On the whole, however, it seems probable that the main acidity reduction takes place on the parts of the mucosa which are directly exposed to the stomach cavity.

Finally, it may be emphasized, that the observation that a buffer inhibits the reduction of the titrable acidity can be taken as a direct support for the view that a back *diffusion* process of the H ions of the secreted "primary" HCl is a dominating mechanism in "regulating" the gastric juice acidity.

SUMMARY

1. It has been pointed out that indirect evidence only is available in favour of the Pavlov hypothesis regarding the "primary" acidity of the gastric juice.

2. A new method is outlined which allows a direct determination of the primary acidity. The principle is based on observations that the rate of acidity reduction is proportional to the hydrogen-ion concentration of the stomach contents (in accordance with the "diffusion theory"). The essential part of the technique is the utilizing of the acid buffering power of glyecol.

3. On cats, stimulated with histamine, reliable values of the primary acidity were obtained only at high rates of secretion. The average value was 208 ± 6 mN., but there are reasons for believing that this value is slightly too high.

4. The possibilities of an "intraglandular" acidity regulation have been discussed.

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THE ACTION IN THE PERFUSED LIVER OF ACETYL-
CHOLINE, SYMPATHOMIMETIC SUBSTANCES
AND LOCAL ANAESTHETICS

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IN 1915 Mautner & Pick drew attention to the action of "shock poisons" such as histamine or peptone on the liver of carnivorous animals. They spoke of a nervous mechanism in the liver producing, under the influence of histamine, engorgement of the whole organ and diminution of the outflow of blood. Later, in 1922, they described histamine as causing a closure of the hepatic veins in the dog, with resulting stasis and dilatation of the liver capillaries, while adrenaline on the other hand by causing constriction in the liver capillaries diminished inflow as well as outflow. The conception of a sluice mechanism in the hepatic veins, closed by histamine, thus introduced by Mautner & Pick received anatomical support from the work of Arey & Simonds [1920], Elias & Feller [1926] and finally Popper [1931]. In 1932, Bauer, Dale, Poulsson & Richards published results of experiments on the perfusion of the isolated livers of different species in a paper in which these anatomical results are summarized, and a general survey of work on the control of the circulation in the liver is given. The observations of Bauer *et al.* were chiefly concerned with the action of adrenaline in the liver of the dog. They found that in the dog there was a sluice mechanism closed by histamine and opened by adrenaline or sympathetic nerve impulses, located near the caval orifices of the main hepatic veins. This variable resistance to outflow was not observed in the liver of the cat, and was weak and inconstant in the liver of the goat. In 1933 Rüegg described experiments in which the dog's liver was perfused by the method of Staub; Rüegg observed, as did Dale and his co-workers, that adrenaline might produce either increased outflow, due to diminished portal resistance, or diminished outflow with rise of portal resistance.

A control of the outflow of blood from the dog's liver by the vagus has not yet been established. Mautner stated that stimulation of the vagus diminished the outflow, and Grab, Janssen & Rein [1929] found that the injection of atropine caused an increase in outflow which lasted for a long time: the observations of these workers were made in dogs under chloralose in which the rate of flow in hepatic artery, portal vein and in the vena cava above and below the liver were recorded. These observations make a vagus control very likely, but Dale and his co-workers were not able to observe an effect of acetylcholine in the perfused liver except in one experiment. Griffith & Emery [1930] found no effect of vagus stimulation in the liver of the cat: similarly McMichael [1933] found no effect of either vagus stimulation or acetylcholine in the cat, but since the evidence of several workers makes it clear that there are differences between different species, these failures do not alter the possibility of a vagus control in the dog.

In the work described below, the method of perfusing the dog's liver given by Bauer *et al.* [1932] has been followed, in order to examine the behaviour of acetylcholine in the first place, and of various sympathomimetic substances in the second place. Thus, for example, Rein [1937] has made special reference to the action of Veritol on the spleen and liver, in both of which organs he states that Veritol causes long-lasting "Entspeicherung" of the contained blood. Various local anaesthetics have also been examined in order to see whether in the liver, as in other organs [Tripod, 1940], they produce sympathomimetic effects.

METHOD

In some respects the exact procedure of Dale and his co-workers has been modified. Thus we have found it preferable to use the lungs of a dog to oxygenate the blood rather than a mechanical oxygenator. In our earlier experiments we used the Hooker apparatus [1915] as supplied by Messrs C. F. Palmer (London) Ltd., but found that often the outflow through the hepatic veins steadily declined, the liver volume became very large and the liver failed to respond to injections of adrenaline at an early stage of the experiment. It looked as though vaso-constrictor substances present in the defibrinated blood interfered with the normal functioning of the liver. When the dog's lungs were used, these substances were removed and a much longer period of reactivity was obtained.

In the perfusion of the liver a cannula is tied into the hepatic artery, through which blood is driven by one Dale-Schuster pump at a pressure which can be regulated; a second cannula is tied into the portal vein

through which blood flows from a reservoir placed at a given height above. In the paper by Bauer *et al.* no actual figures for the height of this reservoir are given; they recorded the lateral pressure in the portal cannula, and used variations in this pressure to indicate variations in portal flow. McMichael [1932] makes the somewhat exaggerated remark that "in perfusion experiments on the isolated liver it is well known that after about half an hour the pressure in the portal vein must be raised to a level comparable to that in the hepatic artery in order to maintain a flow through the organ". We have ourselves determined the pressure in the portal vein of a dog anaesthetized with nembutal by putting a cannula in the splenic vein, and we found that the pressure was between 7-14 cm. water. We have accordingly taken care in our perfusion experiments to see that the portal reservoir was so placed that the lateral pressure in the portal cannula did not exceed 14 cm. water, and was sometimes less than half of this; we tried to work with the lowest possible portal pressure. Thus the comment of McMichael was quite inapplicable to our experiments.

The vessel in which the liver was placed, in the experiments of Dale and his colleagues, consisted of a zinc tray supported by a wide rim of thick ebonite, the outer part of which rested on a copper tank used as a thermostat. A glass lid resting on the ebonite covered the liver above so that, when all joints were sealed with plasticine or putty, changes in the liver volume could be recorded. We found difficulty in obtaining an efficient seal by this method, and constructed a similar apparatus made in one instead of three pieces. The ebonite ring was discarded, and a copper tray for holding the liver was soldered to the outer copper tank by a suitable copper rim. Since, as in the experiments of Dale and his colleagues, the liver rested on muslin supported above the bottom of the tray, it had no actual contact with copper. In all other respects the perfusion apparatus was the same as that described by Bauer *et al.* The perfusion was carried out with defibrinated blood from the two dogs which were used, which gave from 1200 to 1400 c.c. To this was added 0.05-0.1 mg. adrenaline, and the perfusion was begun through the lungs alone until the liver was ready.

EXPERIMENTAL RESULTS

The course of the perfusion

The time which elapsed between the arrest of the normal circulation through the liver and the beginning of the perfusion varied from 10 to 30 min., the usual interval being 15 min. The perfusion through the liver was first begun through the hepatic artery with a small pump stroke.

about 1.5 on the scale. The lid of the plethysmograph was then at once put on so that the liver volume could be recorded. If the volume was observed to be increasing, the stroke of the pump was diminished until the volume remained constant or even decreased; a very slight turn of the screw was sufficient for this. The portal flow was then slowly begun by opening a screw clip on the tube leading from the reservoir to the portal cannula; the outflow at once increased, and for the first minute or two the volume increased also. If there was a large persistent increase of volume, the height of the portal reservoir was dropped or the flow was diminished by the screw clip. By one or other of these means it was possible to maintain the perfusion with a slowly diminishing venous outflow, and a slowly rising liver volume and portal pressure during 30-45 min. During this period the progressive change observed seemed to us to be due to the loss of the normal vascular tone, or to the slow disappearance of the vaso-constrictor substances not yet removed by the lungs which, at the beginning of the perfusion, simulated the effect of this tone. The diminution of outflow, accompanied as it was by a rise of portal pressure, indicated that an increasing resistance in the portal circulation was the main change which was occurring, and at this stage, 25-30 min. after the beginning of the perfusion, the injection of 5 μ g. adrenaline into the hepatic artery produced the prompt increase in outflow, diminution of liver volume and portal pressure illustrated in Fig. 1*a* which has already been described by Dale and his co-workers, and also by Rügge.

A consideration of this observation led us to the idea that a normal condition of perfusion could be maintained for a longer period if an infusion of adrenaline was added at a uniform rate to the reservoir of oxygenated blood, from which the hepatic artery and portal reservoir were supplied. We therefore added adrenaline (e.g. 1 in 200,000) in this way, at rates which varied from an initial figure of 0.03 mg. per min. to a later rate of 0.01 mg. per min. The result was, as we expected, a great increase of venous outflow due to an increased portal flow, as shown by the fall in pressure in the portal cannula. At the same time there was a large diminution in the liver volume, but very little change in the resistance in the hepatic artery.

We regarded the effect of the adrenaline infusion as replacing the vascular control normally exerted on the liver in the body and presumably necessary for its proper function. With such an infusion, judging from our results, it is possible to maintain a satisfactory portal flow without raising the portal pressure, and to avoid engorgement of the liver for relatively long periods.

Effect of adrenaline

Tschernogoroff & Popoff [1936] have perfused the liver of the dog *in situ*, and have described the action of adrenaline as being a diminution in liver volume accompanied by an increase in portal pressure; they make

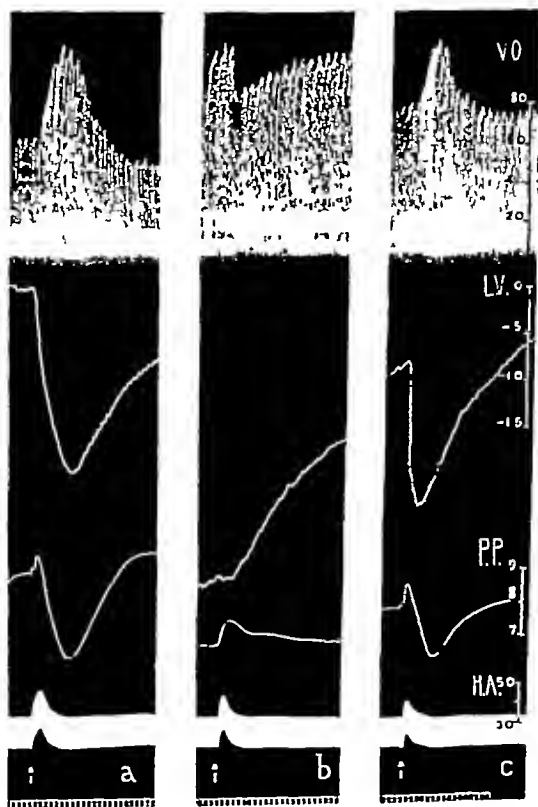


Fig. 1. The records from above downwards are V O, venous outflow, c.c. per min., recorded by Gaddum's outflow recorder; L.V., liver volume recorded by bellows recorder; P.P., lateral pressure in portal cannula in cm. water; a fall in this indicates increased portal flow through the liver, H A, pressure in hepatic artery, in mm. Hg. In *a* is shown the effect of $5 \mu\text{g.}$ adrenaline injected into the hepatic artery when the liver is swollen and the portal resistance high. In *b* is shown the action of the same dose of adrenaline during the infusion of adrenaline (0.25 mg. in 1100 c.c. blood during 14 min.) when the liver volume had become very small and was slightly increasing. In *c* a repetition of *a* after 36 min. when the rate of adrenaline infusion was much less.

no mention of the increased portal flow and increased venous outflow which Dale and his co-workers suggest as the characteristic effect (see Fig. 1*a*). It is true that Bauer *et al.* describe a diminution in venous

outflow accompanied by diminished portal flow, but they observed this in response to large doses of adrenaline, and their paper leaves the impression that they did not regard it as the physiological effect of small doses. Our own observations suggest that the two kinds of response are not due to differences in dosage, but to the conditions. In Fig. 1*b* is shown the action of the same dose of adrenaline as was injected in Fig. 1*a*, namely 5 μ g.; between *a* and *b* an infusion of adrenaline had been made into the blood, so that 0.25 mg. had been added to 1100 c.c. blood during 14 min. The effect of the infusion, during the period between Fig. 1*a* and *b*, was to increase the venous outflow, diminish the liver volume, and diminish the resistance to portal flow. At the point of Fig. 1*b* the liver volume had ceased to decline and was once more slightly increasing, and the injection of adrenaline accelerated this increase: the main changes were, however, the diminution in venous outflow and accompanying diminution of portal flow shown by the increased pressure in the portal cannula. We may note that even so much adrenaline as was infused did not affect the pressure in the hepatic artery. In the period between Fig. 1*b* and *c* the rate of infusing adrenaline was greatly diminished, and in consequence the venous outflow decreased and the portal pressure rose. After 36 min. the record Fig. 1*c* was obtained showing a similar response to that in Fig. 1*a*. We have been able to illustrate the effects of adrenaline in another way, namely by recording the changes during the beginning of an adrenaline infusion into the reservoir of oxygenated blood. In Fig. 2*a* the introduction caused an increase in outflow, diminution in liver volume and decreased portal resistance. In Fig. 2*b*, taken from

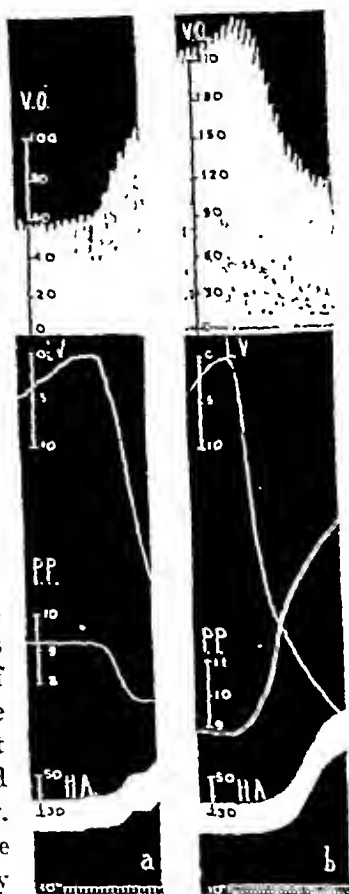


Fig. 2. The records are the same as in Fig. 1. *a* shows the effect of the gradual addition of adrenaline to the blood when there followed an increase in venous outflow and fall in the pressure in the portal cannula. *b*, taken from another experiment, shows the effect of a similar addition of adrenaline when there followed a diminution in outflow, and a rise of pressure in the portal cannula.

another experiment in which even in the absence of adrenaline the venous outflow was very large, the beginning of the adrenaline infusion caused a similar reduction of liver volume but a large rise of portal resistance accompanied by a diminution in venous outflow and a fairly large rise of pressure in the hepatic artery.

In Figs. 1 and 2 there appears to us to be evidence of three adrenaline actions. In Fig. 1*a* and *c* and in Fig. 2*a* the main effect is the removal of a resistance on the venous side, i.e. the opening of the sluice mechanism of Dale and his co-workers. In Fig. 2*b* the second action of adrenaline appeared as an increase of resistance on the inflow side due to constriction of the small vessels coming from both the hepatic artery and the portal vein. We do not think that the decrease in portal inflow was secondary to the rise in arterial resistance, because the former lasted much longer than the latter. The third action of adrenaline, shown in Fig. 1*b*, in which the liver volume was already small due to the adrenaline infused, is shown as an increase of resistance on the outflow side with increase of liver volume. The possibility of obtaining these different responses explains the diverse findings of Clark [1928], Schwiegl [1932] and Rügge [1933], together with those already discussed.

The action of acetylcholine

Since we found that the action of adrenaline in opening the hepatic veins and increasing the outflow was best seen when the perfusion had continued for some time so that all vasotonins and traces of adrenaline had disappeared from the circulating blood, it occurred to us that it might be possible to observe the action of acetylcholine in the opposite circumstances, namely when the perfusion had recently begun and when as a result of the presence of adrenaline in circulation the portal flow was large. We found that this was so, and obtained results like those shown in Figs. 3 and 4. In Fig. 3 the pressure in the hepatic artery was higher than usual, and the injection of acetylcholine in a dose of 10 μ g. into the hepatic artery produced a dilatation of the artery, accompanied by swelling of the liver, an increased resistance to portal flow and diminished output. This picture is precisely the reverse of that produced by adrenaline in Fig. 1*a*, and it was tempting to suppose that the mechanism was precisely the reverse, being mainly the production of an increased resistance to the venous outflow, or in other words, a closure of the sluice. Against this, however, was the repeated observation that, unlike adrenaline or histamine, acetylcholine had no action when injected into the portal vein; it was difficult to understand why an action on the

effluent veins should occur only when the injection was made into the artery. We were, therefore, driven to explain the action of acetylcholine as due to an increased arterial inflow leading to expansion of the liver volume. The swelling of the liver lobules must have diminished the portal



Fig. 3.

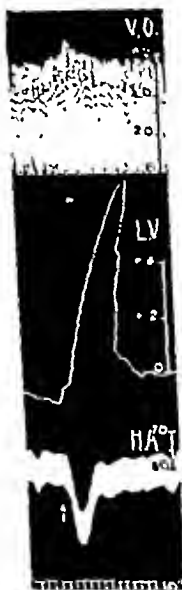


Fig. 4.

Fig. 3. Records as in Fig. 1. To show the effect of injecting 10 μ g. acetylcholine into the hepatic artery. See text. The injection of the same dose into the portal cannula was without effect.

Fig. 4. Records as in Fig. 1 except that the portal circulation was arrested and there is no record of pressure in the portal cannula. The injection of 5 μ g. acetylcholine into the hepatic artery produced the changes shown. See text.

flow as shown by the increase in portal pressure and by the diminution in outflow. Support for this explanation was given by one experiment illustrated in Fig. 4 in which the liver was perfused by the hepatic artery only; there was no portal flow; the injection of 5 μ g. of acetylcholine caused an increase in venous outflow, and not a diminution as in other

experiments. There was a sharp rise in liver volume, and had this been due to a closure of the hepatic veins, an increased outflow could not have been seen. Here the increased arterial inflow could not diminish the portal flow, and therefore the swelling of the liver was greater than usual and accompanied by an increased outflow.

The effect of sympathomimetic substances

We have examined the action of tyramine, ephedrine, Sympatol (or synephrine), Veritol and Benzedrine in that condition of the perfused liver in which adrenaline produces increased portal inflow and increased

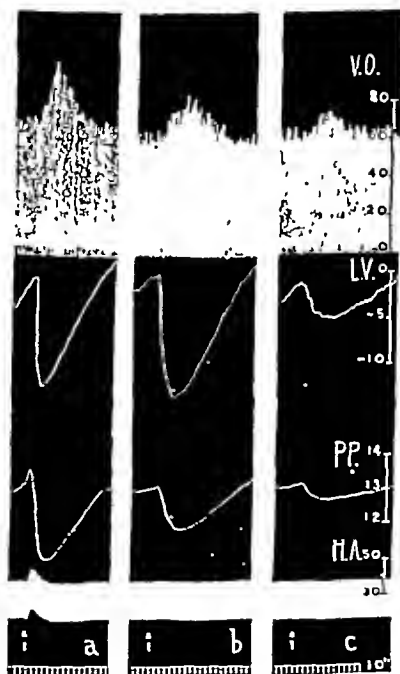


Fig. 5. Records as in Fig. 1. In *a*, 2 μ g. adrenaline, in *b*, 0.1 mg. tyramine, in *c*, 0.1 mg. ephedrine injected into the hepatic artery.

venous outflow with accompanying decrease of liver volume. All these substances were found to have the same effect as adrenaline, though in different degrees. Fig. 5 shows in *b* the effect of 0.1 mg. tyramine injected into the hepatic artery; the change produced on the liver volume was similar in magnitude to that produced by 2 μ g. adrenaline, though the increase in the portal flow was less. Fig. 5*c* shows that 0.1 mg. ephedrine had decidedly less action than the same dose of tyramine.

The most surprising effect was that obtained with Veritol (β (*p*-oxyphenyl)isopropylmethylamine). As already mentioned, Rein [1937] has called attention to the extent to which it leads to a removal of blood from the abdominal viscera, and we have observed (Fig. 6*b*) that it produces a very large diminution in the liver volume in the perfused

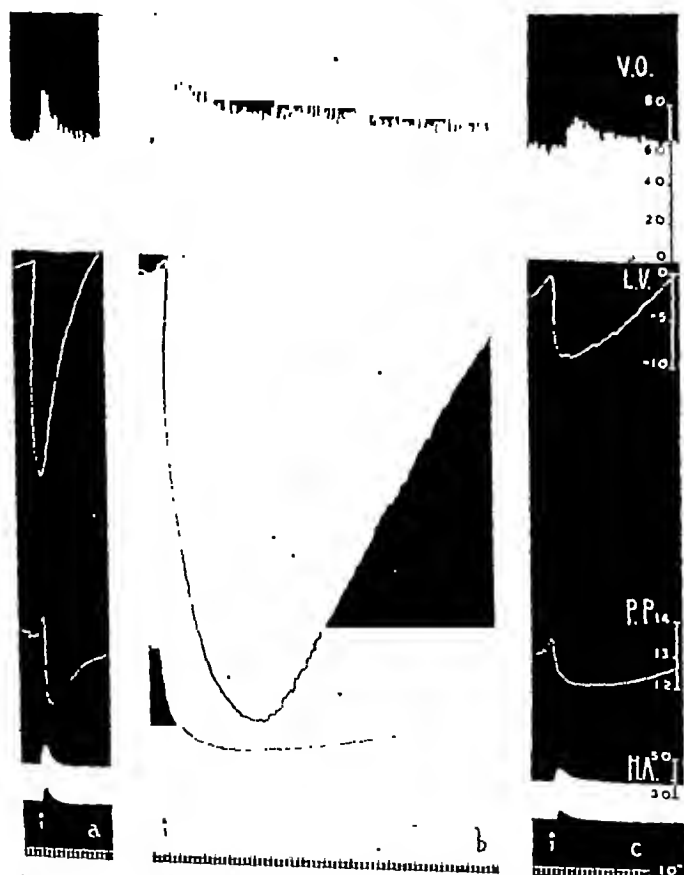


Fig. 6. Records as in Fig. 1. In *a*, 2 μ g. adrenaline, in *b*, 0.5 mg. Veritol, and in *c*, 1 mg. Sympatol injected into the hepatic artery.

liver. In the experiment illustrated the injection of 0.5 mg. led to a reduction of volume of 48 c.c., the initial weight of the liver being 375 g.; thus the liver volume was probably reduced by more than 10%, and the venous outflow was increased from 60 to 90 c.c. per min. In the same experiment, Fig. 6*c*, the effect of Sympatol (which has the same side-chain as adrenaline but only one —OH in the *p*-position) was much

less; we do not, however, wish to convey that the effects in this experiment represent the relative activity of the two substances, for in other experiments Sympatol appeared to have not less than one-third of the action of Veritol.

Benzedrine (β -phenylisopropylamine) was found to have a similar but very prolonged action, as shown in Fig. 7*b*, in which the effect of 1 mg. is recorded side by side with that of 2 μ g. adrenaline (a). When

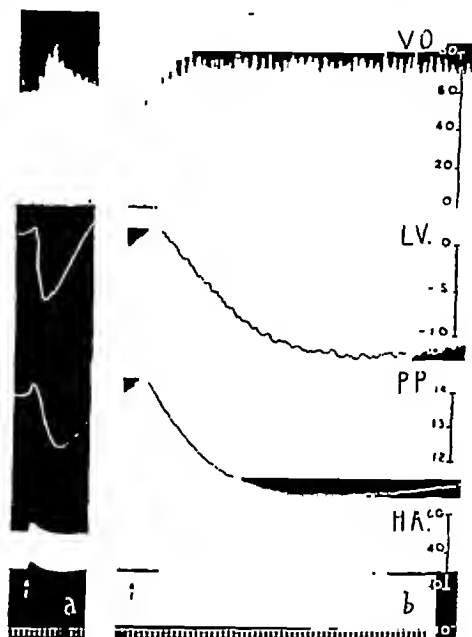


Fig. 7. Records as in Fig. 1. In *a*, 2 μ g. adrenaline, in *b*, 1 mg. benzedrine injected into the hepatic artery.

the perfusion was carried out so that the action of adrenaline was a diminution of portal inflow and of venous outflow, the action of all these substances remained on the whole adrenaline-like.

The action of local anaesthetics

Recently one of us (J.T.) has obtained evidence that the local anaesthetics, butyn, percaïne and stovaine, exert certain adrenaline-like effects. Cocaine has long been known to have an action of this kind, and Maegregor [1939] has observed that procaine also exerts such an action. We have, therefore, examined these five local anaesthetics in the perfused liver, and have found that both cocaine and stovaine clearly produce the same

changes as adrenaline. In Fig. 8 is shown the effect of 5 mg. stovaine (*b*) compared with that of 2 μ g. adrenaline (*a*). The stovaine record is almost indistinguishable from the adrenaline record, except that the arterial constriction is smaller. There is the same increased portal inflow and

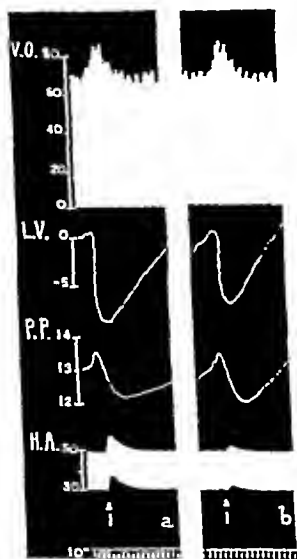


Fig. 8.

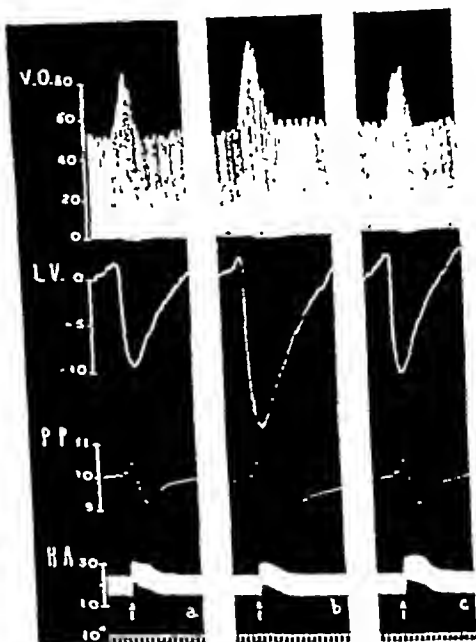


Fig. 9.

Fig. 8. Records as in Fig. 1. In *a*, 2 μ g. adrenaline, in *b*, 5 mg. stovaine injected into the hepatic artery.

Fig. 9. Records as in Fig. 1. In *a*, 10 μ g. adrenaline injected into the hepatic artery. Between *a* and *b*, 10 mg. procaine was injected into the hepatic artery. *b* shows the increased effect of the same dose of adrenaline as in *a* 3 min. after the injection of procaine. *c* shows the disappearance of the potentiation 12 min. later.

increased venous outflow, accompanied by a similar diminution of liver volume. It should be noted that, like the effect of adrenaline, the effect of stovaine is transient and, therefore, not to be explained by a paralysis of the sluice mechanism. The effect of cocaine resembled that of stovaine; a dose of 1 mg. cocaine produced a smaller effect than 5 μ g. adrenaline. Percaine and procaine had a very slight effect only, and butyn had no effect of its own except when injected in a dose of 10 mg., which caused a prolonged increase of portal inflow, venous outflow and liver volume, all probably to be attributed to a paralytic action.

These three substances, butyn, percaïne and procaine, all potentiated the action of small doses of adrenaline. This increase of the adrenaline effect is shown for procaine in Fig. 9. In Fig. 9a is recorded the result of injecting 10 μ g. adrenaline in the hepatic artery; thereafter 10 mg. procaine was injected, and after 3 min. the effect of the same dose of adrenaline was greater than before on venous outflow, on liver volume and on portal inflow. After a further period of 12 min. the original effect of the dose of adrenaline returned as shown in Fig. 9c. In other experiments 5 mg. butyn or 2 mg. percaïne produced a similar temporary potentiation of the adrenaline action.

SUMMARY

1. In the perfused liver of the dog we have found evidence for three effects of adrenaline: (a) opening of the hepatic veins with increased portal inflow and diminished liver volume; (b) increase of resistance due to constriction of the small vessels from the hepatic artery and portal vein; and (c) in the presence of pre-existing adrenaline, an increased resistance on the outflow side.

2. When observations are made early in the perfusion and when adrenaline is present in the blood, acetylcholine injected into the hepatic artery causes an expansion of liver volume, probably due to increased arterial inflow; the expansion of the liver diminishes the portal flow and the net effect on outflow is a diminution. The action of acetylcholine in causing diminished outflow and expansion of the liver cannot be regarded as a closure of the hepatic veins, since it does not occur when acetylcholine is injected into the portal cannula. When there is no portal flow, the output is increased.

3. Tyramine, ephedrine, veritol, sympatol and benzedrine all act like adrenaline, though their effects were more prolonged.

4. The local anaesthetics stovaine and cocaine both have a typical adrenaline-like action. Butyn, percaïne and procaine have little or no direct action of their own, but all of them potentiate the action of adrenaline.

We wish to express our thanks to Prof. J. H. Burn for his help and advice, and to Mr H. W. Ling for his great technical assistance in carrying out the perfusions.

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EFFECTS OF CHANGES IN DIETARY CALCIUM
ON NEUROMUSCULAR TRANSMISSIONBY G. L. BROWN AND A. M. HARVEY¹*From the National Institute for Medical Research,
Hampstead, London, N.W. 3**(Received 3 August 1939)*

IN a previous paper [Brown & Harvey, 1938] we have recorded certain peculiarities in the responses of the leg muscles of fowls to nerve stimulation. We found that when two maximal motor nerve volleys followed one another at intervals less than 150 msec. the action potential of the muscle in response to the second was greater than the response to the first. This effect was shown to be due to the fact that a single nerve volley failed to excite all the fibres of the muscle, but left behind it a period of raised excitability at the motor end-plates, so that a second volley, arriving during this period, could excite the fibres which failed to respond to the first volley.

In the course of another investigation, we had occasion to examine, myographically and electrically, the muscles of a kid, which had been kept for some months on a diet deficient in calcium. The electromyograms from this animal showed effects of essentially the same nature as we had previously observed in the fowl. Since we had not controlled the diets of the fowls we had used in our previous experiments, and since, in any case, it was not improbable that they might have been deficient in calcium, we decided to put the matter to the direct test, and to determine what effects large changes in calcium intake had upon the response of the muscle to nerve stimulation. We are putting on record the results we obtained from the solitary experiment on the kid, as they are in such close agreement with those on the fowls, and supplement them in several directions.

¹ Fellow of the Rockefeller Foundation.

METHODS

The first experiment was done on a female kid, 8 months of age, weighing 12 kg., which had been on a diet deficient in calcium for 4 months. During this time it had shown no increase in weight, and at the time of the final experiment the blood calcium stood at 6.8 mg./100 c.c. as compared with the usual value of about 10 mg./100 c.c. for normal animals. There was severe muscular weakness, and the jaws, long bones, and hooves showed deformities. A full account of the diet, and of the chemical and morphological changes which it produces in goats is given by Glock, Murray & Glover [1939].

For the experiments on the chicks, 18 brood mates were taken 10 days after hatching. Six were fed on a diet adequate in calcium and phosphorus, six received a diet deficient in calcium and high in phosphorus, in its essentials the same as that given to the kid, and the remainder a diet with excess calcium and low phosphorus. After 10-14 days the chicks on the low calcium diet became weak, and were unable to stand or to move normally. We found it desirable to give them a small dose of calciferol about 4 days before the final experiment, in order to keep them sufficiently strong to withstand anaesthesia.

The methods used for electrical and myographic recording were the same as those previously described [Brown & Harvey, 1938, 1939], with the exceptions that we used nembutal (0.2-0.4 c.c. of a 0.6% solution given intramuscularly) as the anaesthetic for the chicks, and a more efficient heater was used for maintaining the muscle temperature at, or about, 40° C.

RESULTS

Goat experiment

Response to single nerve volleys. The tension response and the electrical response of the tibial group of muscles to a single nerve volley were not obviously different from the normal. The tension maximum attained by the isometric twitch was low (0.75 kg.), but not much less than would be expected from such a small animal. Two normal goats of 16.5 and 16.8 kg. body weight had given twitches of 1.3 and 1.1 kg. respectively. The electrical responses with either belly-tendon leads or concentric needle electrodes showed no sign of repetitiveness.

Response to double nerve volleys. When the muscle was excited by two maximal nerve volleys set up at intervals of less than 200 msec., the action potential accompanying the second response was always greater

than the first potential. This phenomenon became much more evident after the muscle had been stimulated 15-20 times. Fig. 1 shows a series of such responses at 12, 40 and 200 msec. apart, and in Fig. 2 the results

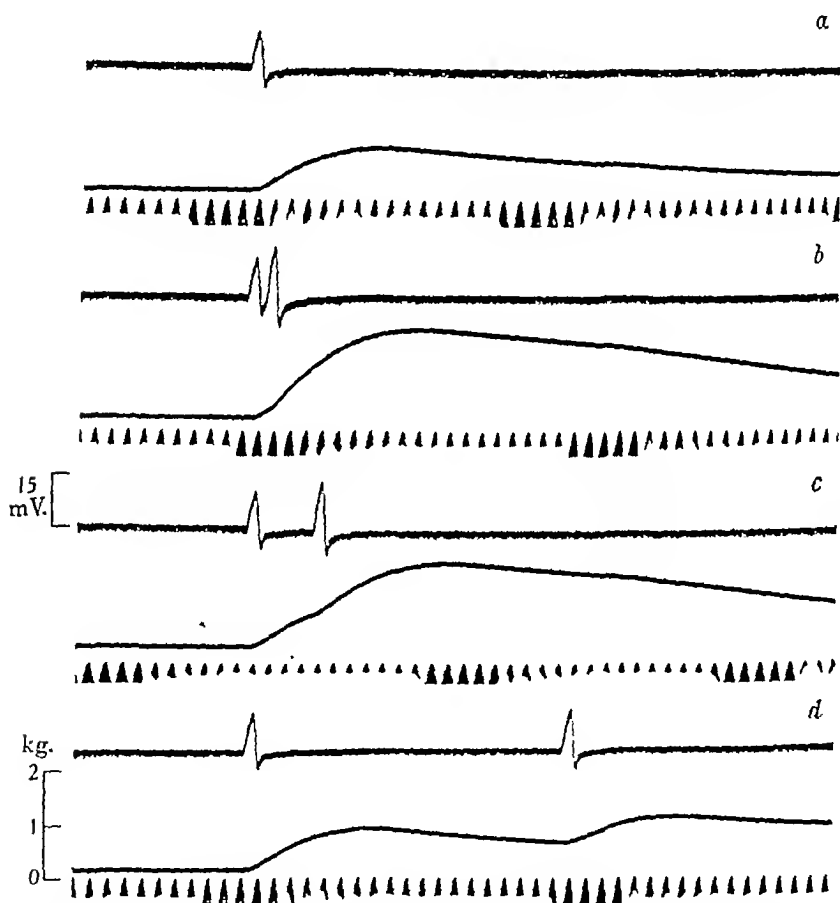


Fig. 1. Goat, low Ca diet. Myogram and action potential of tibialis group in response to: (a) single maximal nerve volley, and to two volleys at (b) 12, (c) 40 and (d) 200 msec. apart. Time 10 and 200 msec.

of the experiment are plotted graphically, together with those of a similar experiment on a normal goat. The increase in the second response is at its maximum when the two potentials become discrete at 12 msec., and the difference then declines gradually until at 200 msec. they are almost equal.

The maximum tension developed in response to two volleys at short intervals was 1.6 kg. The ratio between the tension responses to single and to paired stimuli was thus within the range recorded in the normal animal.

We took care to exclude the possibility that the phenomenon was due to local effects in the nerve. The stimuli were usually applied through paired electrodes with the cathodes 1.8 cm. apart. Increase of stimulus strength, reversal of the sign of both shocks, application of both shocks through the same electrodes. delivery of the shocks from the same or

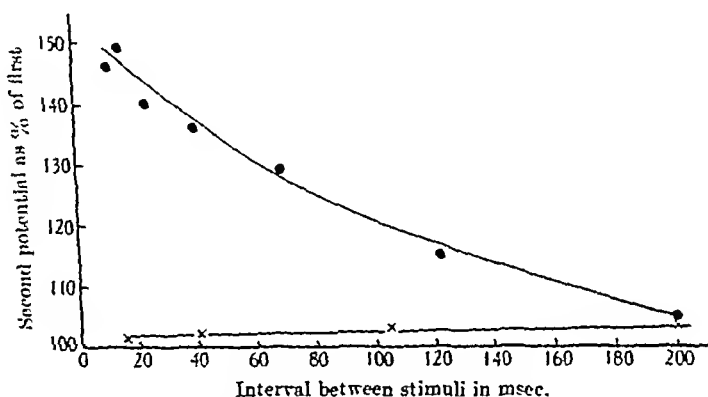


Fig. 2. Curve showing relation between size of second response and interval between stimuli applied to nerve in the Ca-deficient (•) and the normal (×) goat.

from two distinct stimulators, and variation in the relative position of the two shocks on the nerve, were all equally without effect on the response recorded from the muscle.

Response to tetanic stimulation of the nerve. At frequencies of stimulation ranging from 40 to 80 per sec., the maximum size of the negative deflexion of the action potential was not attained until the fifth or sixth response (Fig. 3). The subsequent potentials remained equal unless the tetanus was of such duration as to lead to the decline normally seen after prolonged stimulation.

A single twitch, elicited within two seconds of a brief period of tetanic stimulation, had an action potential equal in size to the fifth or sixth response of the preceding tetanus, and a tension some 60% greater than that of a single twitch immediately preceding the tetanus. The usual disparity between the action potentials of the first and second of two closely following responses was absent when the two responses were elicited within 1 min. after a period of tetanic stimulation of the nerve. In these circumstances both potentials of the pair were equal to the fifth or sixth

of the preceding tetanus, and the summed tension was greater than that of a similar paired response before the tetanus. If closely timed pairs of stimuli were given to the nerve at regular intervals after a tetanus, the gradual return of the muscle to its pretetanic condition could be followed; each successive pair of responses showed a progressive decline in amplitude of both components, but the decline of the first of the pair outstripped that of the second, so that the characteristic disparity gradually reappeared.

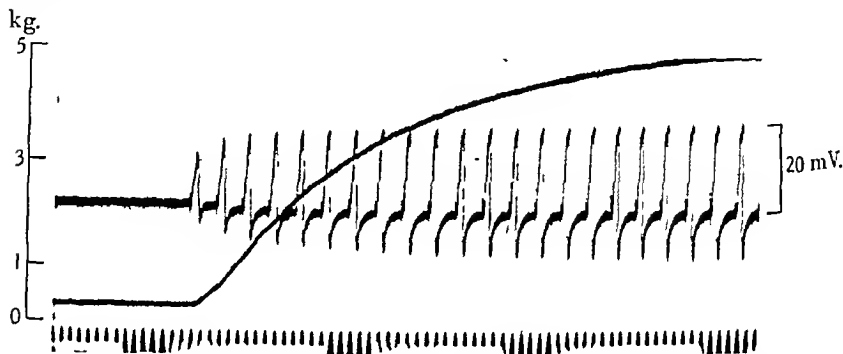


Fig. 3. Goat, low Ca diet. Myogram and action potentials of tibialis group responding to maximal stimulation of nerve at 37 per sec. Time, 10 and 200 msec.

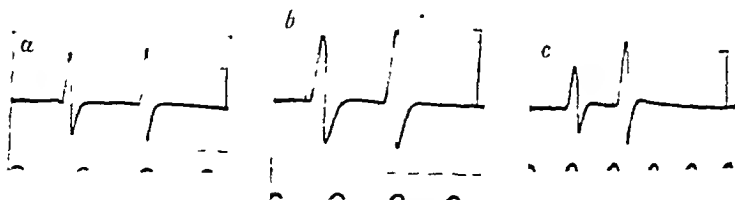


Fig. 4. Action potentials of gastrocnemius of chicks. Response to two maximal nerve volleys at 25 msec. apart. (a) Normal Ca diet; (b) high Ca diet; (c) low Ca diet. Calibrations show (a) 25, (b) 25 and (c) 7.5 mV. Time 20 msec.

Experiments on chicks

Normal and high calcium diet. In these chicks the peculiarities of neuromuscular transmission were as evident as in the fowls which had been used in our previous investigation. For instance, in the chicks on a normal calcium diet the second action potential of a pair, set up 25 msec. apart, was in one experiment 15%, and in another 19% greater than the first (Fig. 4a), and the difference in size of the two potentials declined as the interval between them was lengthened. With tetanic stimulation, the second electrical response was greater than the

first, but subsequent responses remained of the same size as the second. Similarly, in two chicks on the high calcium diet, the second responses at an interval of 25 msec. were 12 and 14% respectively of their first responses (Fig. 4*b*). The responses to tetanic stimulation were again not significantly different from our previous observations.

The serum calcium values in these chicks were as follows: normal diet, 10.1 and 10.1 mg./100 c.c.; high calcium diet, 12.0 and 11.2 mg./100 c.c.

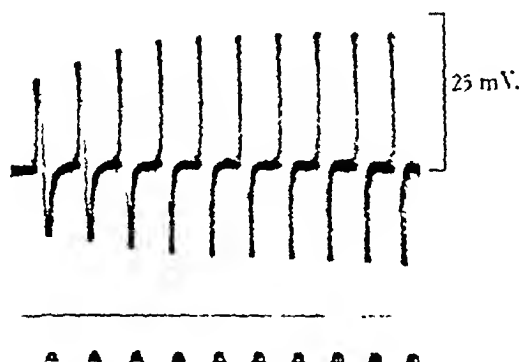


Fig. 5. Action potentials of gastrocnemius of chick on low Ca diet. Maximal tetanic stimulation of nerve at 55 per sec. Time 20 msec.

Low calcium diet. As was to be expected, these chicks showed, in an exaggerated form, the deficiency in transmission of a single volley normally seen in the fowl, and the responses were very like those already described for the kid. In one experiment, the second potential was 54% larger than one preceding it by 25 msec. (Fig. 4*c*). The disparity gradually diminished as the stimuli were separated in time, and the two responses were approximately equal when 200 msec. apart.

With tetanic stimulation, the progressive increase in amplitude of the successive deflexions was very striking (Fig. 5); in one experiment the maximum was not attained until the eighth stimulus, after which the responses remained constant in size.

The serum calcium values in the birds used were 8.5, 5.9, and 9.1 mg./100 c.c.

DISCUSSION

It is sufficiently clear from the experiments on the chicks that the peculiarities in neuromuscular transmission in the fowl, which we previously described, were not the result of calcium deficiency, but are

apparently a normal feature of the process in these animals. It is, however, equally evident that they can be noticeably exaggerated by a chronic deficiency in calcium in the food. In the kid, the effects of the calcium deficiency are more striking since the mammal shows no suggestion of such a phenomenon in normal circumstances. The transmission process in the calcium-deficient animals bears some resemblance to that observed in one in which transmission has been partially blocked by curarine [Brown, 1938]. In both, the second of a pair of responses close together is greater than the first, and in both, transmission is partially, though temporarily, restored by tetanization of the motor nerve at a suitable frequency. There, however, the resemblance ends; in a curarized preparation the third, and subsequent responses of a series decline rapidly in amplitude, whereas the increase of amplitude in the calcium-deficient animal may proceed for eight or more responses. This progressive decline of successive responses in a curarized animal is probably attributable to the summation of the prolonged period of depression which follows, and probably curtails the much shorter lasting period of augmented second response. This period of depression would appear, therefore, to be absent in the response of the calcium-deficient muscle, and this suggests that calcium lack is a different process from simple curarization. Our present information is, however, insufficient to allow us to come to any definite conclusion. It is not improbable that the facilitation process, which follows the arrival of a nerve impulse at the neuromuscular junction, is intimately associated with movements of potassium ions [Brown & Euler, 1938] and it is, therefore, likely to be modified by changes in the calcium content of the tissues. A further complication is introduced by the fact that changes in calcium may affect the liberation of acetylcholine. Feng [1937] has suggested that acetylcholine liberation may be increased by increase in calcium concentration, and Harvey & MacIntosh [1940] have found that perfusion of a ganglion with calcium-free Locke's solution completely abolished the discharge of acetylcholine by pre-ganglionic nerve stimulation, and with it the synaptic transmission of the excitatory effect. The changes in neuromuscular transmission evoked by chronic deficiency in calcium present a picture of some complexity, but it is probable that a further study of muscles in this condition will be of value in elucidating some details of the transmission process.

SUMMARY

1. A kid, kept on a diet deficient in calcium, showed a defect in neuromuscular transmission, such that a single maximal motor nerve volley failed to elicit a maximal response from the muscle.

2. With repeated stimulation, at a sufficiently high frequency, each successive response of the muscle became greater until transmission was fully restored at the fifth or sixth response.

3. Chicks, on a similar diet, showed the same phenomenon, which is an exaggeration of the defect normally seen in the fowl.

4. A diet rich in calcium did not eliminate the normal peculiarity in neuromuscular transmission in the fowl.

We wish to thank Mr R. E. Glover for providing us with the kid, and for controlling the diets of the chicks.

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SUMMARY

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THE OXYGEN SUPPLY OF THE FOETAL BRAIN OF THE SHEEP AND THE EFFECT OF ASPHYXIA ON FOETAL RESPIRATORY MOVEMENT

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(Received 5 August 1939)

THE object of the present research is primarily to gain information upon the oxygen conditions under which the brain lives and develops during pregnancy. This involves the analysis of samples of the blood going to and coming from the brain; for the former we have used the carotid, for the latter the blood from the superior longitudinal sinus or the torcular Herophili. Each of these samples has further interest. The comparison of the carotid blood with the blood from the umbilical artery and the umbilical vein respectively gives additional information on the much vexed question of the degree of mixing in the foetal heart of the streams of blood which arrive thereat. The interest which attaches to the blood leaving the brain expands in the direction of studying the effect on the activity of that organ when the oxygen level is lowered. In particular it has been established that the activity of the brain is subject to a degree of inhibition [Barcroft & Barron, 1939] which commences about the 50th day of foetal life and is thoroughly established by the 60th day. The seat of this inhibition is subcortical and has been located primarily in the upper part of the forebrain, though the centre may be rather long and "tail off" into the lower part of the forebrain and even into the midbrain. The inhibition can be abolished by asphyxia. It seemed important to find out at what degree of asphyxia the inhibition was suppressed.

The existence or otherwise of movements of the respiratory type forms a good criterion of the degree of inhibition ruling in the brain. The problem therefore from the experimental point of view reduces itself to this: Pinch the umbilical cord and at what oxygen level in the sinus blood is respiratory movement released?

TECHNIQUE

So far as the carotid is concerned, there is nothing to be added to the description of the technique given by Barcroft, Kennedy & Mason [1940]. With regard to the collection of samples from the sinus, it is possible, in the younger foetuses in which the brain case has not hardened, to obtain the blood by simple puncture. Whilst this method has much to recommend it from the point of view of rapidity and reduction of trauma to the minimum, it has one disadvantage, namely the uncertainty that the blood obtained is precisely that from the sinus and nothing else. There are two sources of contamination: (1) the rupture of some small artery in the pia mater, which does not often occur, and (2) the admixture of the blood with cerebrospinal fluid, which is a very frequent, and indeed almost constant occurrence when the blood is collected by puncture. The evidence of this is that the blood sample has a lower, sometimes a much lower, oxygen capacity than that of samples taken simultaneously from blood vessels. It is best, therefore, even for samples taken before the closing of the fontanelle, to remove the structures which cover the brain, if necessary under a local anaesthetic.

Table I gives values for the oxygen in carotid and sinus bloods during pregnancy.

TABLE I

Oxygen in carotid blood					Oxygen in posterior sinus blood				
No.	Foetal age days	Content c.c./100 c.c. of blood	Capacity c.c./100 c.c. of blood	Saturation %	No.	Foetal age days	Content c.c./100 c.c. of blood	Capacity c.c./100 c.c. of blood	Saturation %
422	62	6.2	9.9	63	—	—	—	—	—
500	63	6.9	9.5	73	—	—	—	—	—
289	76	10.2	13.0	78	—	—	—	—	—
340	78	9.1	10.9	88	—	—	—	—	—
519	80	8.2	10.7	76	—	—	—	—	—
286	88	9.8	14.6	67	425	86	2.1	13.6	15
312	89	9.3	13.4	69	424	93	5.8	16.4	35
331	96	9.0	13.4	67	490	93	7.6	14.3	53
484	97	9.9	15.1	66	432	101	7.0	16.7	42
		8.9	11.9	75	479	101	9.4	16.0	59
		8.2	11.9	69	—	—	—	—	—
327	105	6.2	14.5	42	488	111	5.4	16.0	34
		7.2	14.5	49	492	120	9.5	16.1	59
459	112	12.4	14.2	87	450	120	6.6	17.1	38
306	113	10.3	14.8	70	504	128	4.4	21.0	21
285	127	10.6	16.3	65	489	128	8.6	21.0	41
165	130	9.4	15.2	62	475	132	6.1	19.8	31
470	139	9.7	21.0	46	—	—	—	—	—
277	139	10.3	16.9	62	—	—	—	—	—
444	143	7.7	18.4	42	—	—	—	—	—
515	143	6.6	15.2	43	—	—	—	—	—
498	144	6.0	18.6	37	491	144	2.5	19.7	15
512	145	6.5	12.7	52	415	146	3.5	22.3	15.7

OXYGEN SATURATION OF CAROTID BLOOD

The values for the carotid are much more regular than in former experiments, which had been carried out under less rigid conditions. Thus with the exception of foetus 327, with which there was no obvious fault to be found technically, though the uterus was rather contracted, the saturation in every experiment prior to the 139th day is over 60 %, between the 63rd and 127th days inclusive the saturation is 65 % or over, whilst only in two experiments is the saturation under 80. After the 130th day the saturation drops and after the 140th day, i.e. within a week of term, it drops markedly. The phrase "within a week of term" raises a point which presents a constant difficulty. In discussing these very late observations it would be much more interesting to know exactly the number of days the foetus was short of birth than the number since conception. Thus taking 147 days as the normal period of gestation, foetus 470 had 8 days still to run, but it may easily have had less, in fact in the case of 512 labour was actually commencing.

OXYGEN CONTENT OF CAROTID BLOOD

The oxygen content of the carotid blood seems to be singularly steady—apart again from foetus 327. Over the period 66–139 days inclusive, there are only two experiments in which the carotid blood contains less than 8.9 vol. of oxygen per 100 vol. of blood and only one in which it contains more than 10.9. This is remarkable, because the oxygen capacity varies to a much greater extent. Compare, for instance, foetuses 470 and 277—the oxygen capacities of the blood are 21 and 16.9 vol. % respectively and the percentage saturations 46 and 62 %, but the oxygen contents of the carotid blood are 9.7 and 10.3 %.

Though we point out this constancy in the oxygen content of the carotid blood we do not stress it. To do so would lead logically to the supposition that some form of regulation existed, which may be the case, but to assume it would be to endow the foetus with properties of which we have no knowledge.

In the last stage of pregnancy the oxygen content of the carotid blood drops. The drop is not so large relatively as that in the saturation on account of the simultaneous increase in the oxygen capacity.

OXYGEN IN BLOOD FROM VENOUS SINUSES

Table I gives figures for the oxygen capacity, oxygen content and percentage saturation of blood coming from the sinuses. These figures are more variable than the carotid figures. The saturation and the content

are on the whole higher about the 100th day than at any other time. As in other cases, there is a marked fall in the last days of pregnancy.

As regards the oxygen difference between the arterial and the venous blood there is little to be said, as the sinus experiments were not on the same animals as those on the carotid. It may, however, be pointed out, though not stressed, that the average of the oxygen contents of the carotid blood between the 86th and 132nd days was 9.3 vol. % while that of the sinus blood over the same period was 6.6 vol. %, a difference of 2.7 vol. %. The data for the 144th-146th days show no reduction in this figure, which seems to suggest that even though at the end of pregnancy the whole level of oxygen determination, both arterial and venous, is lower than in the middle, the brain is not being starved.

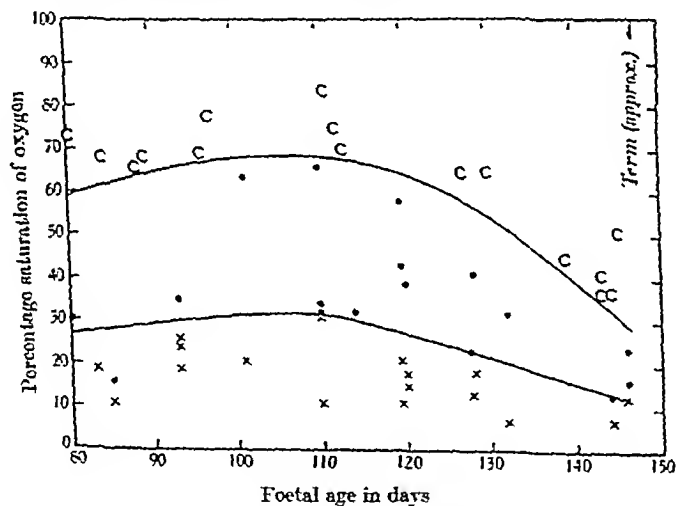


Fig. 1. Percentage oxygen saturation of blood of foetal sheep; c = in carotid artery; • = in venous sinuses of brain, no respiratory movements occurring; x = in venous sinuses of brain when respiratory movements had appeared, after pinching the cord.

To pass now to the exploration of the oxygen level at which the respiratory mechanism is released from the inhibition which controls it. In the experiments which have just been described the foetus was in every case quiescent so far as respiratory movement is concerned, but in many cases after the blood had been drawn in the manner described, the umbilical cord was occluded, usually by pinching gently between the finger and thumb, and when respiratory movements had appeared another sample was taken. It was not always possible to obtain the sample at the very moment of the first breath, but in Fig. 1 all the

determinations marked \times were taken when the foetus was exhibiting respiratory movements, whilst those marked \bullet were taken whilst it was not doing so. The line of demarcation appears to be extraordinarily sharp.

But while the line is well defined it is not at a constant level throughout the whole of pregnancy, it tends to fall towards the end; in other words, from the 110th day onwards the cells of the inhibitory centre in the diencephalon become increasingly resistant to asphyxial conditions in the blood. This may be either because their inherent resistance is increased or because the supply of blood becomes more copious. Another and even more interesting point is that the margin between this release line and the line separating the saturation of carotid blood from that of sinus blood becomes narrower as pregnancy approaches, so that by the 146th day the actual conditions under which the foetus lives are very close indeed to those at which respiratory release takes place, whilst at the earlier stages a very ample margin exists.

It is of interest to note that Lennox, Gibbs & Gibbs [1935] found that unconsciousness in man appeared pretty regularly when the oxygen in the blood of the internal jugular vein fell to 24 % saturation.

THE RELATION OF THE CAROTID BLOOD TO THAT IN THE UMBILICAL VESSELS

In the determinations with which we have dealt it has been the object to get the samples as soon as possible after the exposure of the foetus. This technique does not as a matter of course lend itself to an exact comparison of the bloods of the carotid and the umbilical vessels, for which purpose the latter may be obtained within a matter of seconds of opening the uterus, while the abstraction of the carotid blood requires some dissection and has never been obtained by us in less than about 5 min. from the first incision through the uterine wall.

Tests therefore have been required to ascertain whether the blood in the umbilical vessels remains of constant composition over say 5-10 min. The answer seems to be that it rarely remains quite constant, but during a great part of pregnancy, if the precautions which we have described are observed, the composition of the blood changes little; not enough to affect the general issue of the comparison between the carotid blood on the one hand and the blood from the umbilical artery and vein on the other. Towards the end of pregnancy this is by no means the case. We have therefore carried out eight experiments in which the blood from the umbilical vessels was collected before and after that from the carotid and

the results plotted graphically against time. The results are shown in Fig. 2.

They indicate:

(1) That the composition of the blood in the carotid artery is always intermediate between that in the umbilical vein and that in the umbilical artery.

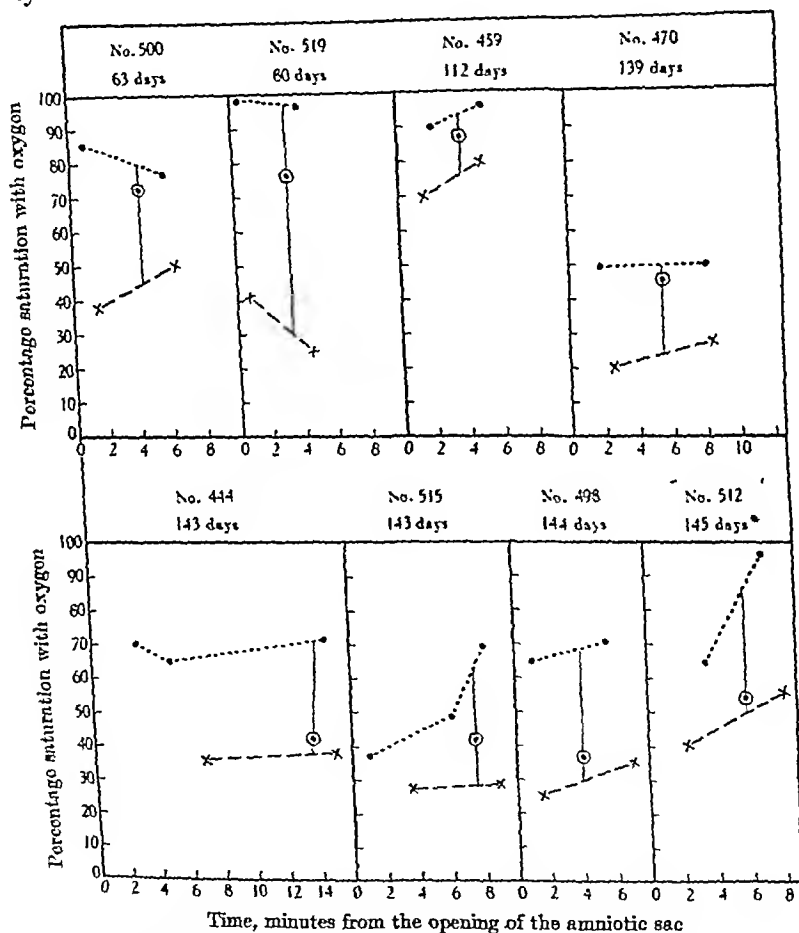


Fig. 2. Percentage oxygen saturation of blood of foetal sheep: • = in umbilical vein; × = in umbilical artery; ○ = in carotid artery; * = commencement of labour.

(2) That over the greater part of pregnancy the carotid blood approximates in composition to that in the umbilical vein, but that in the last few days it often approaches that in the umbilical artery.

So far as the information prior to 130 days goes, the results of six experiments in which there was a simple comparison of the samples is given in Table II. The samples in each experiment were probably all

TABLE II

No.	Foetal age days	Oxygen in umbilical vein		Oxygen in carotid artery		Oxygen in umbilical artery	
		Content c.c./100 c.c. of blood	Per-centage saturation	Content c.c./100 c.c. of blood	Per-centage saturation	Content c.c./100 c.c. of blood	Per-centage saturation
340	78	10.1	93	9.1	88	3.4	31
255	83	10.3	75	6.9	50	6.6	48
286	88	13.5	93	9.8	67	5.8	40
312	89	11.7	87	9.3	68	4.1	31
306	113	12.9	88	{ 9.0 10.3	70	8.9	60
285	127	12.5	77	10.6	65	9.5	58

taken within 10 min. of one another but in no definite order. In one young foetus (83 days) the carotid blood was of the same composition as that in the umbilical artery; otherwise they show that the carotid oxygen is intermediate between the oxygen in the bloods of the umbilical vein and artery and markedly different from both.

A word must be said in comparison of the present results with those of Huggett [1927] on the one hand and Kellogg [1930] on the other. The values which they obtained may be summarized as follows:

	Huggett			Kellogg		
	Umbilical vein	Carotid	Umbilical artery	Umbilical vein	Left ventricle	Right ventricle
O ₂ content c.c. O ₂ per 100 c.c.	8.0	5.9	2.9	2.86	2.43	2.38
O ₂ saturation % approximate*	50	38	18	14	12	12

* Calculated on basis of O₂ cap. for goats, 16; for dogs, 20.

Except in the case of the umbilical artery (Huggett), *vis-à-vis* the right ventricle (Kellogg), Huggett's figures are markedly higher than those of Kellogg. Our own are higher than both, markedly higher than both until the last week; even then they are on the whole higher than Huggett's and definitely higher than Kellogg's. It must be remembered that these authors aimed at having their foetuses at a late stage of development, so that the difference between their results and ours may not be so great as appears.

The classical view of the circulation through the foetal heart, put forward by Sabatier [1791], that there was a complete crossing of the streams of blood entering by the superior and inferior vena cava is by no

means universally held. The degree of crossing is discussed elsewhere [Barcroft, 1938]. Huggett's results indicate a great degree of "crossing of the streams" in the heart, the carotid blood coming from the left ventricle containing much more oxygen than that in the umbilical artery coming via the dorsal aorta from both ventricles but largely from the right. Of the ten experiments quoted in this paper on foetuses younger than 140 days all except one (Table II, sheep 255) are in obvious agreement with Huggett's result: but when we come to those performed on sheep at 143-145 days that is not the case. In these the carotid blood approximates more or less in composition to the blood in the umbilical artery. This could be interpreted as indicating a rather complete though as yet imperfect degree of mixing in the heart. The point is still obscure, however, because the blood in the umbilical vein is not that which reaches the heart by the inferior vena cava; the latter is a mixture of the umbilical blood and the blood which comes from the trunk and legs. Take as an example foetus 498 (see Fig. 2); $7\frac{1}{2}$ min. after the amniotic sac is opened, i.e. when the carotid sample is taken, the saturation of the blood in the umbilical artery appears to be 28. This is the blood which feeds the trunk; if the tissues of the trunk reduced that blood by 18 %, it would return 10 % saturated. The blood in the umbilical vein is 62 % saturated. Now if one part of blood at 10 % saturation were mixed with two parts at 62 % the resultant blood would be 45 % saturated, which is very near to the actual saturation in the carotid. Without further data then we cannot apportion the extent to which the drop in the percentage saturation of the carotid blood just before birth is due to inefficient mixing in the heart, and to what extent it is due to relative increase in quantity and/or venosity of the blood from the trunk as compared with that from the placenta. The ciné X-ray finds of Barclay, Barcroft, Barron & Franklin [1939] support the latter view.

SUMMARY

1. A comparison is drawn between the oxygen content and saturation of the carotid blood with that in the venous sinuses of the brain. This comparison gives the superior limit and probably the inferior limit of the oxygen in the capillaries of the brain. The carotid saturation up till the last week of pregnancy is of the general order found in the arteries of persons at very high altitudes. In the week before birth there is a marked drop.
2. Occlusion of the cord induces movements of the respiratory type; these seem on grounds stated elsewhere to be due to "release" from an

inhibitory centre in the forebrain. The oxygen in the blood of the venous sinuses gives a very clear index of the degree of asphyxia necessary to produce this release. It does not of course prove that the cause is oxygen want rather than CO_2 excess, or a combination of the two, but it does serve as a good index.

3. The blood from the carotid is always intermediate in its oxygen content between that in the umbilical vein and that in the umbilical artery. Till within a few days of birth it is nearer to that in the umbilical vein (in 9 cases out of 10 quoted). In the few days before birth the carotid oxygen drops relatively to the others and they all drop absolutely. The bearing of this drop in the carotid oxygen on the circulation in the heart is discussed.

We wish to thank Dr M. F. Mason and Dr J. A. Kennedy for their assistance in experiments quoted in this paper between the serial numbers of 250-360.

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OXYGEN IN THE BLOOD OF THE UMBILICAL VESSELS OF THE SHEEP

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A NUMBER of authors have published particulars concerning the oxygen content and percentage saturation of the blood in various vessels in the mammalian foetus. These results have been characterized by the very great divergence in the values obtained. This divergence applies even to the observations of any one observer and to any one vessel.

The records have fallen into three main categories, concerned respectively with (1) the comparison of the maternal and foetal bloods, (2) the metabolism of the foetus and (3) the comparison of the foetal carotid blood with that in the umbilical vessels.

Though the errors likely to appear in the records vary a little according to the object for which the experiments are performed, we may for the present purpose consider them together and indeed it is only necessary to take a single vessel, the umbilical vein, to exemplify the divergence which applies to all (Table I).

TABLE I. Oxygen in blood in umbilical vein

Author	Animal	Content vol. %	Percentage saturation. The figures in brackets are approximate, cal- culated from an assumed oxygen capacity: goat = 16, dog = 20
Cohnstein & Zuntz [1884]	Sheep	6.3	42
Barcroft, Flexner & McClurkin [1934]	Goat	8.5-3.9	81-(33)
Roos & Romijn [1938]	Cow	9.8-6.3	90-53
Haselhorst & Strom- berger [1930, 1931]	{ Man (normal birth) { Man (Caesarean)	14.9-5.3 5.7-2.5	45-33
Eastman [1930]	{ Man (in utero) { Man (at birth)	13.3 average 13.2-8.2	63 50
Huggett [1927]	Goat	10.5 average	
Kellogg [1930]	Dog	12.5	(80-31)
Steele & Windle [1939]	Cat	11.8-2.1 8.6-0.3	(60-11) 75-(3)

The statements from this laboratory on the subject of the oxygen in the umbilical vein have been very guarded. A curve was given by Barcroft, Elliott, Flexner, Hall, Herkel, McCarthy, McClurkin & Talaat [1935] which showed a gradual rise from 60 to 87 % saturation in the weeks between the 70th and 115th days, with an abrupt fall in the last week. Our own comments on this curve at once show our caution and the reason for the present research. "None of our observations has given us more solicitude than those of direct records of the percentage saturation of blood taken from the umbilical vein. The results vary greatly but Fig. 1 gives the upper limit. Often the points are much lower. At present we have not sufficient information to discuss fruitfully whether these lower points are due to experimental procedure, e.g. the constriction of the uterine vessels when the uterus is opened." But while it is suggested above that the lower points are due to experimental error, it does not follow that the higher ones are free from it. Thus as may easily be demonstrated, if the maternal circulation in the placenta is good and the foetal circulation is restricted, the blood leaving the placenta along the umbilical vein will become bright red even if it were not so before. In that case the blood in the umbilical artery will be correspondingly dark and the oxygen difference great.

TECHNIQUE

Our experiments have been done on sheep. The principal source of error seemed likely to be the exposure of the cotyledons to something different from their normal environment. It was therefore decided to draw the samples if possible without taking the foetus out of the uterus.

This proved to be easy in the case of the umbilical artery and the umbilical vein, especially when dealing with singlets. In such cases the blood from both horns of the uterus contributes to the general flow, and through an incision in the wall of the horn not occupied by the foetus blood may be taken from branches of the main artery and vein. There is as a rule no difficulty in obtaining blood from the carotid, or from the jugular vein without taking the foetus out of the uterus. The secret lies in fixing the head, otherwise the foetus is very elusive. On inspection of the uterus, before any cut is made in its wall, it is often possible to locate a projection caused by an ear. A stitch is put through the wall swiftly, and the pinna is sewn to the uterus. The wall is then cut open over what is judged to be the position of the jugular vein, the skin of the foetus is opened in the same position and the lips of the two wounds are clipped together so that in effect the uterine wall becomes part of the foetal in-

tegument. A cut is made along the posterior edge of the jugular, then by suitable pressure from below (after a little practice) there is no difficulty in forcing up the carotid into the wound so made.

When as in our later experiments the mother is under a spinal or local anaesthetic, the above operation on the foetus may be done under a local anaesthetic. Up to the hundredth day or thereabouts the above precautions suffice, but after that date another factor becomes very important, namely the position of the uterus. The foetus has obtained a weight of upwards of a kilo. and it is very easy to interfere with the maternal circulation by undue pressure on the larger vessels. Therefore the paramount object must be to keep the uterus in its normal position. For instance, if to obtain carotid blood it becomes a choice between moving the uterus and withdrawing the head of the foetus, the latter would be the procedure.

The observation of these precautions offers no great difficulty until after about 130 days; then the whole operation becomes so fraught with pitfalls that, even after doing it many times, we never approach it without the feeling that we are at best facing an almost impossible task.

The reason why the operation becomes so difficult is of course that the operator is in competition with the inherent physiology of the animal. It is part of the very essence of intra-uterine life that at its termination the uterus and the foetus should react to the most trivial interference in a way similar to birth, i.e. primarily by occlusion of the uterine vessels and the cord. The umbilical cord itself becomes much more responsive to touch, to strain, or even to exposure. At an earlier stage, the dropping upon it of a few drops of 1 % formalin will usually suffice to preserve the calibre of the vessels, not so as birth approaches. It is necessary at this stage that the cord should never be pulled, that it should never be out of an isotonic medium and, if possible, that it should not be touched.

What is true of the cord is true of the maternal vessels though in a less degree. The procedure which we have found most successful for this final stage has been as follows: The sheep under a spinal, or perhaps better under a local anaesthetic, is placed in the bath. A V-shaped incision is made from the pubic symphysis; in one direction it goes up the middle line, in the other parallel to and along the abdominal side of Poupart's ligament. The length of these incisions is a matter of judgement, the object being to expose the uterus without exposure of other abdominal viscera; the edge of the omentum is often a good guide. The sheep is now turned over on to its side in the bath. To facilitate this the animal is lying on slings; two for its support pass underneath it, while the

third, which is for the purpose of keeping its back hard up against the wall of the bath (so as to give as much room as possible between the abdominal wall and the side of the bath adjacent to it), is passed round the sheep but with both ends brought over the side of the bath against which the back is pressing. A small stand or bed for the foetus to lie upon is suspended from a support which can at once be put in position. The position is along the abdominal wall, at the level of the middle line. The height of the sheep is regulated with the slings so that it is sufficiently, but not more than sufficiently, immersed in the saline (40° C.). The uterus is then opened. At this stage, especially in animals of more than 140 days foetal age, there is little bleeding. The foetus is withdrawn and placed, without subjecting the cord to any strain, on its stand. Notwithstanding these precautions there is always the fear of the aeration of the blood becoming less efficient as the time passes and it is always well to take the samples as soon as possible. In the case of the umbilical vessels this should be within about a minute of opening the uterine wall. In the case of the carotid, the dissection needs about 5 min., but of that more will be said in a future paper.

We use any good syringe, but the needle demands care, the larger the bore of the needle the quicker the samples can be obtained, the smaller the bore the less the trauma. Our principle has been to take the smallest needle consistent with the withdrawal of the blood in about 3 sec. In very young foetuses the blood should be withdrawn more slowly lest the general pressure relations, especially the circulation in the heart, be upset; a useful syringe for the purpose is one of which the plunger screws out. The point should be very sharp. Among the methods of damaging the cord, one of the most effective is the endeavour to pierce its wall with a needle, the point of which is not of a high degree of sharpness. Of course, the smaller the needle relatively to the lumen the less the interference with the blood flow. The blood samples were analysed in the Van Slyke manometric apparatus, making use of Mason's [1939] technique for the delivery of small samples. Usually 0.2 c.c. were taken for analysis. The possible errors introduced by using such samples are small and completely negligible as compared with the physiological errors caused by manipulation and the loss of blood in the case of animals younger than 80 days.

The sample when taken is at once transferred to a small container in which is heavy liquid paraffin and under that an anti-coagulant, either in the form of liquid or powder. It is important that this anti-coagulant should kill the blood, i.e. that it should inhibit the glycolytic power of the

blood [Evans, 1922] and so destroy the power of the blood to reduce itself. When a solution was used, it had the following composition:

(COOK) ₂ H ₂ O	3 g.
NaF	1 g.
NaOH M/100	1 ml.
Water to	100 ml.

When the powder was used, it consisted of a mixture of sodium fluoride and potassium oxalate in the proportion of 1 : 3 by weight. Of the powder 5–15 mg. were taken per c.c. of blood, more being required for younger foetuses. As the blood can only be stirred slowly lest it should emulsify with the paraffin, traces of heparin powder were also added.

The containers were kept in ice till the analysis could be made.

The only series of observations in which an attempt has been made to follow the oxygen content and percentage saturation systematically throughout pregnancy has been carried out in this laboratory on sheep and some of the results have been published [Barcroft, 1938]. These results indicate a general tendency, as pregnancy proceeds, for the percentage saturation to fall in the umbilical artery, the umbilical vein and the carotid artery. The figures obtained were however very irregular; for instance, two foetuses on which the determinations were carried out at approximately the same period of pregnancy gave the following results:

Sheep	Foetal age days	Oxygen saturation (%)	
		Umbilical vein	Umbilical artery
19	137	42	10
27	138	75	43

If in the placenta the maternal flow was unduly slow and the foetal flow was not, one might expect a result such as that shown by sheep 19 in which the foetal blood was poorly oxygenated. If on the other hand the foetal flow was unduly slow and the maternal flow normal, the foetal blood would probably arrive at the placenta very dark but would leave it very bright, and such cases have been observed. It is not to be assumed that because the blood in the umbilical vein is bright red that the conditions are satisfactory. Such independent alterations in the relative flows of the maternal and foetal bloods through the placenta may be physiological and it is not unreasonable that so great a degree of irregularity should exist; on the other hand before accepting it, it seemed desirable to carry out observations under much stricter conditions.

The object of the present paper is to supply records of the saturation and content of the blood in the umbilical and other vessels, which inspire more confidence than those hitherto published, more confidence because obtained under controlled conditions.

THE OXYGEN IN THE UMBILICAL VESSELS

The question we set ourselves was the following: Of a number of determinations taken at different stages of pregnancy and widely different in result which, if any, could we duplicate by the improved techniques which we have described?

In general, the answer was that the greater the precautions taken the more likely we were to duplicate observations in which both the oxygen in the umbilical vein and that in the umbilical artery were high. This at least is so up to about 130 days. (The period of gestation of the sheep is 147 days.)

Table II gives figures for oxygen content percentage saturation, and oxygen capacity of the blood of sheep fetuses up to 145 days.

TABLE II. Oxygen in blood in umbilical vessels

Sheep no.	Foetal age days	Oxygen content c.c./100 c.c. blood		% saturation		Oxygen capacity c.c./100 c.c. blood
		Umbilical vein	Umbilical artery	Umbilical vein	Umbilical artery	
500 S	63	8.0	3.6	85	38	9.4
299 T	66	10.8	2.3	83	18	13.0
340 S	78	10.1	3.4	93	31	10.8
519 S	80	11.4	4.7	98	42	11.6
255 S	83	10.3	6.6	75	48	13.8
286 S	88	13.5	5.8	93	40	14.5
312 S	89	11.7	4.1	87	31	13.4
331 S	96	13.8	9.4	92	62	15.1
459 S	112	12.9	10.0	87	67	14.9
262 S	113	11.4	8.0	83	58	13.7
306 S	113	12.9	8.9	88, 87	60	14.8
345 S	126	8.4	5.9	77	54	10.9
285 S	127	12.5	9.5	77	58	16.3
470 T	139	10.6	4.3	50	20	21.2
444 T	143	12.8	6.2	70	34	18.4
515 S	143	8.6	4.8	48	27	18.0
498 S	144	12.35	4.8	65	25	19.0
572 T	145	8.5	5.3	65	40	12.21

S=singlet; T=twin.

The table seems to show:

(1) That up till near the end of pregnancy (somewhere between the 127th and the 139th day) it has been possible to obtain duplicate readings

from different sheep for the percentage saturation of oxygen in the blood in the umbilical vein with considerable success.

(2) That in this period the percentage saturation is high, using the word high to mean of the same order as has been found in the arterial blood of men who have been conscious, though not necessarily alert. Somewhere in the region of 70 % may be taken as a useful line.

(3) That on the whole the percentage saturation of the blood in the umbilical vein is highest between the 75th and 100th days, after which it gradually falls.

(4) That during the last week of pregnancy the percentage saturation in our experiments drops considerably and the figures become irregular. Some reasons will be given later for the supposition that these irregularities may not be entirely due to our inability to compete with the admittedly difficult conditions of the experiments.

(5) That the percentage saturation in the umbilical artery underwent greater changes than in the umbilical vein. Starting at about 65 days at a quite low figure, it rose at about 95-115 days to values in the region of 60 % saturation, then again fell.

(6) That the oxygen difference expressed in terms of percentage saturation was great in the younger foetuses being of the general order of 50-60 % saturation. It then decreased and from the 96th till the 127th day it was only about 20 % saturation. In the final week it was irregular but it never approached the 60-80 day values.

(7) That apart from one or two anaemic sheep (572 and 345) the oxygen capacity per c.c. of blood followed the course described by Elliott, Hall & Huggett [1934] for the goat, namely it rose in the early stages, remained fairly level from the 80th day until close upon term, when it again rose abruptly.

(8) That the oxygen content of each cubic centimetre of blood in the umbilical vein is more irregular than the percentage saturation; the oxygen content is maintained to the end of pregnancy, the rise in oxygen capacity and the fall in percentage saturation more or less balancing one another.

(9) That the oxygen content of each cubic centimetre of blood in the umbilical artery starts at a low figure in the 60 day stage, rises to a high one at about 100 days and then again sinks.

(10) That the absolute oxygen difference is large in the earliest periods, frequently 7 or 8 c.c./100 c.c. of blood, and drops to 3-4 c.c. between 96 and 127 days. In the final period it is variable.

DISCUSSION

The first question which arises for discussion is: How do the observations of previous workers on the oxygen in the blood of the umbilical vessels appear in the light of the work described above?

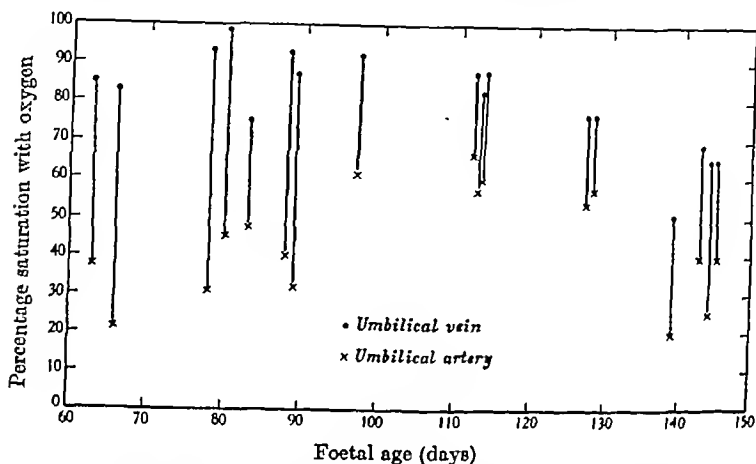


Fig. 1. Percentage saturation with oxygen of blood in umbilical vessels during foetal life.

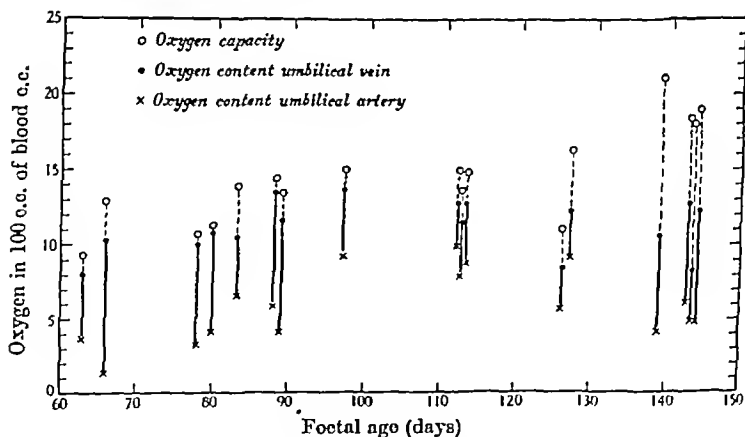


Fig. 2. The oxygen capacity and oxygen content of blood in the umbilical vessels during foetal life.

The answer is perhaps most easily obtained by inspection of Figs. 1 and 2. Comparing Table I with those figures, except perhaps in the case of Kellogg's [1930] determinations, it would be possible to find at some stage of pregnancy a determination in the figures which matched any one

in the table. Therefore unless the foetal ages are specified the determinations mean little. On the other hand most of the values given in the table aimed at being near term and as such are probably not far wrong, for at that time the results seem to be inherently variable, the variation being associated, as Steele & Windle [1939] have pointed out, with alterations of tension in the uterine walls.

Where previous observations fall short is that none of them does or can give a picture of the "oxygen in the umbilical blood during pregnancy". The oxygen in the blood during pregnancy undergoes a series of ordered changes which can only be placed in a single picture by the plotting of serial determinations made under very carefully controlled conditions.

Inasmuch as the saturation in the blood of the umbilical vein is, till close upon term, over 75 % and up till 113 days is usually in the region of 85-95 %, the new information gives a very different idea from that given by Table I.

The composition of the blood which supplies the head will form the subject of a later paper but there is no controversy about that which supplies the body; it is identical with that found in the umbilical artery. Till the 90th day the saturation of this blood is low and as the oxygen capacity is only about 10 vol. %, the oxygen content is low also. Comparing the blood at this period with that in the umbilical artery at term, the saturations are of the same order but the content at term is greater owing to the greater oxygen capacity.

A striking feature is the sudden rise in the oxygen in the umbilical vein in both saturation and content at about the 90th day. It is difficult not to associate the rise with certain events which take place in the placenta at that time:

(a) The cotyledons attain their maximum weight at that time.

(b) The vascular bed in the placenta undergoes a remarkable development about that time. The observations of Barcroft & Kennedy [1939] showed that between about the 80th and the 93rd day the vascular bed in the placenta increased threefold, whilst by the 93rd day the bed was already of the same order of size as at term; the maximal size of the bed seemed to be between the 120th and 130th days (Table III).

It is perhaps premature to pursue this phase of the subject further, pending observations as yet unfinished on the structure of the placenta and the vascular conditions.

TABLE III

Volume of blood

Number	Foetal age days	Volume of blood		
		Total c.c.	Foetus c.c.	Placental bed c.c.
268 S	78	53	43	10
340 S	78	52	29	23
264 T	82	53	31	22
266 S	93	136	65	71
333 S	105	220	150	70
298 T	108	{180	{114	66 R
		{189	{120	69 L
309 S	111	266	165	99
256 S	112	287	193	94
343 S	118	219	126	93
289 T	124	326	198	128
337 S	125	289	206	83
345 S	126	319	141	178
348 T	133	444	349	95
273 S	140	565	498	67

S=singlet; T=twin; R=right; L=left.

SUMMARY

The information in the paper is summarized in the numbered paragraphs on pp. 352-3.

We are indebted for help to Dr Barron, Mr P. H. Forsham, Mr Cowie and Capt. MacDonald. We wish to thank the Medical Research Council for a grant which was partially expended on this research and the Cambridge University Department of Agriculture for their kindness in undertaking the fertilization of the sheep.

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EFFECT OF ACETYLCHOLINE, PROSTIGMINE, POTASSIUM AND FATIGUE ON THE CROSSED EXTENSOR REFLEX AND ON ITS REFLEX INHIBITION IN THE TOAD

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SCHWEITZER & WRIGHT [1937] found that intravenous injection of a large dose of acetylcholine inhibits the knee jerk in cats. The present communication is concerned with the effects of acetylcholine, prostigmine, potassium and fatigue on reflex inhibition. The contralateral extensor reflex in the toad and its homolateral inhibition served as type reactions.

METHOD

After division of the spinal cord the toads were allowed to recover by being kept at 2-3° C. for 1-2 days. The reflexes were recorded as isotonic contractions of the gastrocnemius muscle. All connexions of the gastrocnemius with the remainder of the animal except the sciatic nerve were cut, including the vessels leading to the gastrocnemius muscle, so that the perfused substances could only affect the central nervous system and could have no peripheral effects.

The preparation was perfused with Ringer's solution through a thoracic aorta at a pressure of 50 cm. Hg, and the test substances were added to the perfusate. The influence of the perfused substances on reflexes due to their vasomotor effects [Schweitzer & Wright, 1937a] were minimized by the relatively high perfusion pressure chosen. Control experiments showed that such a high pressure had by itself no qualitative effect on the reflexes.

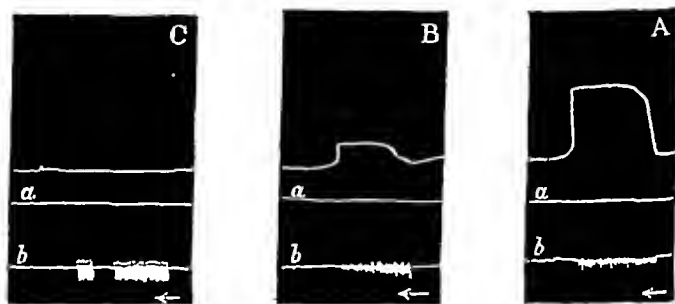
The contralateral extensor reflex was evoked by stimulating the central end of the contralateral sciatic nerve. It was inhibited by stimulating the central ends of two intra-abdominal branches of the

homolateral sciatic nerve. The nerves were stimulated by induction coils leading to silver electrodes. The contralateral nerve was stimulated at a frequency of 6-10 per sec., and the homolateral nerve at a frequency of 50 per sec. The intensities of the currents were just above the threshold values and were the same before and after addition of the drugs to the perfusion fluid.

RESULTS

The experiments described have been carried out on 102 toads. Fig. 1-3 are typical records.

The contralateral extensor reflex is essentially the same in the Ringer perfused preparation (Fig. 1 A) as in one with a normal circulation. The



The contralateral extensor reflex.

Fig. 1 A. Toad perfused with Ringer.

Fig. 1 B, 1 C. Toad perfused with acetylcholine. *a*, homolateral stimulation; *b*, contralateral stimulation.

addition of 0.01 % of acetylcholine to the perfusate, however, diminishes (Fig. 1 B) or abolishes (Fig. 1 C) the response. This effect of the drug can be partly overcome by increasing the intensity of stimulation.

The homolateral extensor reflex is also diminished or abolished by acetylcholine.

The contralateral extensor reflex and its homolateral inhibition. The crossed extensor reflex can be inhibited by stimulation of the sciatic nerve on the same side as shown in Fig. 2 A. This inhibition is produced only when the homolateral stimulus is relatively weak; if it is increased beyond a certain value its effect is reversed and summation results. The inhibiting and summing ranges of intensity depend on the strength of the contralateral reflex stimulus, being increased with increase of the latter. The summation phenomenon is shown in Fig. 2 B, the height of the summed contraction returning to that of the original response when the homolateral stimulus ceases.

In the presence of 0.01 % acetylcholine, the crossed reflex being diminished or abolished, the effect of homolateral stimulation is to counteract the inhibition produced by the drug and to evoke a large reflex response (Fig. 2C). This response continues after cessation of the homolateral stimulation, for the whole duration of the contralateral stimulation, but after an interval the contralateral stimulation applied alone again evokes only the diminished response.

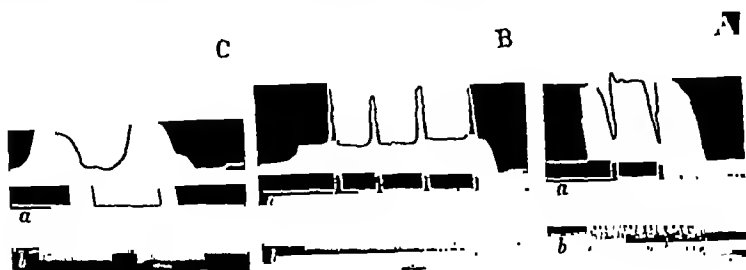
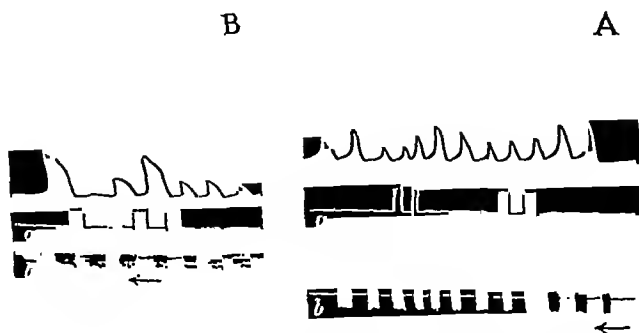


Fig. 2A. Toad perfused with Ringer. Contralateral extensor reflex and its homolateral inhibition.

Fig. 2B. Toad perfused with Ringer. Contralateral extensor reflex and summation caused by a strong homolateral stimulation.

Fig. 2C. Toad perfused with acetylcholine. Contralateral extensor reflex and homolateral summation.



The influence of homolateral stimulation on subsequently evoked contralateral extensor reflex

Fig. 3A. Toad perfused with Ringer.

Fig. 3B. Toad perfused with acetylcholine.

The influence of homolateral stimulation on a subsequently evoked contralateral extensor reflex. In normal or Ringer perfused spinal toads a series of stimuli evoke a series of contralateral extensor reflex responses

of the same height. If in a pause between two such stimuli a homolateral stimulus be interpolated and the latter is of such an intensity as would evoke no response by itself but would inhibit the crossed reflex if applied concurrently, the immediately subsequent response to the contralateral stimulus is not affected or may be reduced (Fig. 3A). In the presence of acetylcholine such an interpolation of a homolateral stimulus in the interval of a series of contralateral stimuli, however, greatly enhances the response to the contralateral stimulus immediately following it (Fig. 3B).

That this effect of acetylcholine was not due to progressive non-specific changes in the properties of the reflexes was shown by the constancy of the responses in the absence of the drugs for a period of several hours, much longer than that occupied by the relevant observations, and by the fact that removal of the drug restored after some hours those properties which were characteristic of Ringer perfusion.

The effect of prostigmine. All the reflex phenomena which have been described above in connexion with the actions of acetylcholine have also been studied under the influence of prostigmine (0.25 mg./100 c.c.) which produced qualitatively exactly the same effects as did acetylcholine.

The effect of potassium. The reflexes of toads perfused with Ringer's solution with a content of potassium chloride 4–8 times normal behaved qualitatively in the same way as in animals with normal circulation.

The effect of fatigue. When in toads with normal circulation or perfused with Ringer the reflexes were fatigued by constant stimulation of the contralateral and homolateral nerves, the reflex responses are replaced by responses comparable in every way to those obtained in fresh animals which have been perfused with acetylcholine or prostigmine (the records obtained are almost exactly the same as Figs. 1C, 2C and 3B). This effect can only be due to the fatigue of the spinal cord because stimulation of the motor nerve to the gastrocnemius still produces a normal contraction.

DISCUSSION

The effect of acetylcholine on the contralateral extensor reflex and its homolateral inhibition in decerebrated cats was studied by Martini & Torda [1939]. The reflexes in cats behaved in a similar manner to the corresponding reflexes in toads.

The behaviour of the reflexes in spinal toads under the experimental conditions described above is shown diagrammatically in Fig. 4.

The essential results of the observations are: first, that both the contralateral and the homolateral extensor reflexes are paralysed by acetylcholine and prostigmine as is the knee jerk in cats; and secondly,

that a homolateral stimulation by a current of an intensity which causes inhibition of the contralateral extensor reflex in animals with normal circulation, antagonizes the paralyzing action of acetylcholine or prostigmine on the contralateral extensor reflex; this effect of the homolateral stimulation persists even after its cessation.

The paralysis can be explained in terms of current views of the role of acetylcholine in reflex mechanism, either by supposing that low concentrations of acetylcholine have an excitatory, high concentrations an inhibitory effect on the spinal cord, or by supposing with Schweitzer &

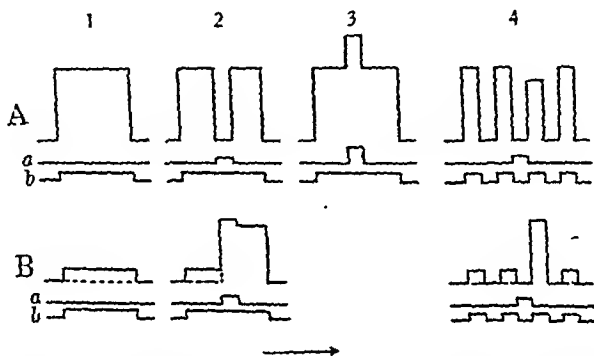


Fig. 4. A. Toads with normal circulation or perfused with Ringer. B. Toads perfused with acetylcholine. (1) The contralateral extensor reflex. (2) The contralateral extensor reflex and its homolateral inhibition. (3) The contralateral extensor reflex and summation with a strong homolateral stimulation. (4) The influence of homolateral stimulation on subsequently evoked contralateral extensor reflex. — Reflexes of most toads. . . . Reflexes of some toads.

Wright [1938] that acetylcholine accumulated within the anterior horn cell causes stimulation, but accumulated outside the cell causes inhibition. The effect of a stimulus which normally causes reflex inhibition in antagonizing the paralytic action of acetylcholine suggests that reflex inhibition may increase the rate of disappearance of the accumulated acetylcholine in some chemical or physical way.

If acetylcholine has any role in the normal reflex mechanism such as by being liberated, on stimulation, at the endings of the nerves in the spinal cord, the antagonism just mentioned seems to indicate that there must be, in addition, some other chemical or physical process. The reason for this supposition is that, as explained below, neither of the two suppositions mentioned in the previous paragraph explain the antagonism observed, even when they are combined with Kato's theory [1934] of the presence of excitatory and inhibitory fibres in the sciatic nerve.

If the first theory were sufficient, an animal injected or perfused with acetylcholine would show, if anything, increased inhibition when the homolateral nerve is stimulated, because this stimulation would liberate acetylcholine; but the opposite has been observed.

On the second theory it must be assumed that the concentration of acetylcholine in the perfused animals is so high outside the cell that it causes inhibition, and that the reflex inhibition by homolateral stimulation is provoked by liberation of acetylcholine outside the cell. Thus in acetylcholine perfused animals the concentration of acetylcholine outside the cell would be increased by homolateral stimulation and reflex contraction, as observed, could not take place.

The effect of fatigue is almost the same as that of acetylcholine and prostigmine, and so it is possible that during fatigue an acetylcholine- or prostigmine-like substance accumulates in the spinal cord.

SUMMARY

1. The influence of acetylcholine, prostigmine, potassium excess and fatigue on the crossed extensor reflex of the toad and on its inhibition by homolateral stimulation has been studied.

2. A weak homolateral stimulus inhibits, but a stronger one augments the contralateral reflex response. Increased intensity of contralateral stimulation increases the strengths of the homolateral stimuli needed to produce these effects.

3. Both contralateral and homolateral extensor reflexes are paralysed by acetylcholine and prostigmine in high concentration.

4. A relatively weak homolateral stimulus antagonizes the paralysing effects of acetylcholine and prostigmine on the contralateral extensor reflex. This effect of the homolateral stimulation has been observed, when it was applied both during the contralateral stimulation and immediately preceding the contralateral stimulation.

5. Fatigue has almost the same effect as acetylcholine or prostigmine.

6. Potassium (4-8-fold normal) makes no qualitative change of the reflexes.

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THE INFLUENCE OF DIURETICS ON THE OSMOTIC WORK DONE AND ON THE EFFICIENCY OF THE ISOLATED KIDNEY OF THE DOG

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THE external work performed by the kidney is almost entirely represented by the osmotic work done in the formation of urine from plasma. The osmotic work which can be calculated is the theoretical minimum work and is independent of the mechanism of formation of urine. It is perhaps the most significant way of summarizing the functions of the kidney as an excretory organ. The osmotic work plays much the same part in renal physiology as does the mechanical work in muscle physiology; asynchronous contraction of muscle fibres and deviation from a longitudinal direction of pull makes it likely that the mechanical work done by individual muscle fibres would add up to something considerably greater than the observed external work done by the muscle; similarly, if we could measure the osmotic work done in different segments of the renal tubule, and suppose that positive work done by the kidney in one segment could not be neutralized by negative work in another segment, we should obtain a value for osmotic work done by the kidney which is greater than the theoretical minimum external work in which such neutralization is presupposed.

The relation between the external work done and the chemical energy degraded in the organ, as indicated by the oxygen consumption, is of interest in two connexions. First, the efficiency so calculated has been found [Borsock & Winegarden, 1931] to be only of the order of 1%—much lower than the maximum efficiency of muscle. This comparison is,

¹ Assisted by a grant from the Medical Research Council.

however, inappropriate, for the work done in forming urine was calculated from analyses of the constituents of a 24 hr. sample, and this should properly be compared with the mechanical efficiency of muscle averaged over a similar period. The maximal efficiency of the kidney is unknown, for there has been no social or scientific incentive to discover the conditions under which it would occur, comparable with that provided for muscle by athletics. Secondly, the distinction between the "mechanical" and "tubular" types of diuresis has been given point by the findings of Barcroft & Brodie [1905*a, b*], Barcroft & Straub [1910] and their followers that only this latter type was accompanied by increase in oxygen consumption or in work done by the kidney. It seemed to us of consequence, therefore, to undertake a more comprehensive analysis of the change in work done during various forms of diuresis, and to discover whether the magnitude of such a change could be used as a trustworthy detector of tubular as contrasted with glomerular activity. Further, since a small change in efficiency would invalidate any conclusions concerning renal mechanisms which might be drawn from changes in oxygen usage, we have observed the simultaneous changes in oxygen consumption of the kidney and in the osmotic work done under various conditions.

Previous estimates of the osmotic work done by the kidney have been concerned first, with the absolute magnitude of the work, for example Borsook & Winegarden's [1931] calculation from the data in the literature giving the constitution of a typical 24 hr. sample of human urine, and secondly, with the increase in work, so far as this can be inferred from estimations of urea and chloride only, produced by "tubular" diuretics, for example the investigations of Glaser, Laszlo & Schürmeyer [1932] on the effects of urea and hypertonic saline on anaesthetized dogs.

Our observations were mainly concerned with discovering the minimum number of analytical procedures which would yield a fair approximation to the total osmotic work, and with demonstrating the changes in the work and efficiency characteristic of both mechanical and tubular types of diuresis. For this purpose the isolated kidney of the dog provides a convenient preparation both because "mechanical" and "tubular" diuresis can be evoked in a more unequivocal and quickly reversible form than in the whole animal, and because the determination of the efficiency of the organ requires simultaneous measurement of its oxygen consumption, which is particularly reliable in this preparation for reasons given by Kramer & Winton [1939].

METHODS

The double pump-lung single kidney preparation of the dog was like that used by Bickford & Winton [1937], with the modification which enables the oxygen content of the arterial and venous blood to be measured and recorded as described by Kramer & Winton [1939].

The latter apparatus was improved in detail as follows. The calibration of the arteriovenous oxygen difference in terms of galvanometer deflexion was rendered even more independent of changes in blood flow by inserting an electrically heated silver tube in the blood circuit between the renal vein and the "oxygen unit", so that the arterial and venous blood could be brought to exactly the same temperature when they were switched on to the unit. The galvanometer recording amplifier was mains' driven; disturbances due to supply voltage variations were eliminated by the use of a push-pull circuit and by various standard voltage regulating devices, which were so effective that even the lamp illuminating the galvanometer mirror and thence the polarized photocell could be heated from the mains. The lamp in the oxygen unit was, however, heated as before by current from a car battery.

Small amounts of urea and creatinine were added to the blood in the perfusion circuit in order to produce concentrations in the serum convenient for the analytical methods used. This initial addition to the blood was made at least an hour before the kidney was connected to the circuit; after connexion, a continuous infusion of a suitable solution of urea and creatinine was adjusted to a rate intended to maintain the serum concentrations constant despite the loss in the urine of amounts varying from time to time.

Chemical estimations

Creatinine was determined as alkaline picrate [Folin, 1914] photo-electrically on the principle suggested to us by Dr Glenn Millikan. Light from a 6 V. 24 W. lamp passed through the test solution in an optical glass trough to two Se-SeO₂ photocells, one protected by a green, and the other by a red colour filter (Wratten nos. 61 and 29), chosen so that creatinine-free picrate solutions transmitted about the same amount of light to the photocells, whereas creatinine picrate affected one cell more than the other. The differential current was passed through a Cambridge "Pot" 1000Ω. mirror galvanometer; the difference between the deflexions corresponding with zero and some arbitrary standard concentration of creatinine was adjusted to yield some arbitrary scale deflexion (400 mm.),

and intermediate deflexions were then calibrated against creatinine concentration. Forty calibration curves were obtained over a period of about 2 years, each with galvanometer readings of 0 and 400 mm. adjusted to fit solutions containing no creatinine and 100 mg. per 100 c.c. respectively. The variation of the curves is shown by the following means (mg./100 c.c.), standard deviations from the mean of a single observation, and numbers of observations: 5 ± 0.13 (4); 10 ± 0.12 (37); 20 ± 0.35 (33); 30 ± 0.50 (21); 40 ± 0.66 (17); 50 ± 0.60 (9). In the region of 10–20 mg. per 100 c.c., about 5 mm. galvanometer deflexion corresponds with a difference of 1 mg. per 100 c.c. of creatinine. Estimations by this method performed without recalibrating on the same day thus involve errors of less than 2%. If, as was the custom in the experiments described below, calibration curves were obtained at the time of making the determinations, the errors are reduced to less than 1%, a high order of accuracy for so quick and simple a method involving only inexpensive apparatus.

Urea in serum was determined by Conway & Byrne's [1933] method, utilizing the stable preparation of urease, which has no ammonia-producing impurities, produced by the method of Van Slyke & Cullen [1914]. *Ammonia in urine* was estimated by the same method.

Urea in urine was precipitated as dioxanthylurea, and oxidized by dichromate, as described originally by Luck [1928] and modified by Lee & Widdowson [1937]. The excess of dichromate was titrated against thiosulphate. The amount of dichromate reduced by the dioxanthylurea is only roughly proportional to the amount of urea; accurate determinations require a calibration chart.

Chloride was estimated by electrometric titration with silver nitrate [Eggleton, Eggleton & Hamilton, 1937], *sulphate* by a modified benzidine method, *electrical conductivity* in the microcell at 500 c.p.s. [Winton, 1936] and *vapour pressure* by the method of Hill [1930].

The calculation of osmotic work done by the isolated kidney

The methods of estimating the osmotic work done in the formation of urine from blood plasma have been reviewed by Cushny [1926] and Borsook & Winegarden [1931]. Since the derivation of the equation by von Rhorer [1905] the methods used have been essentially the same. Von Rhorer calculated the total work as the sum of the partial osmotic works done in concentrating or diluting each of the constituents of the plasma. He showed that the partial osmotic work done in producing

n mol. of a substance at a concentration C_u in the urine from a concentration C_b in the plasma is

$$nRT \left\{ \frac{C_b - C_u}{C_u} - \ln \frac{C_b}{C_u} \right\},$$

where R is the gas constant and T the absolute temperature. We have employed this formula for unionized substances. For univalent ions, however, it can be shown that the partial osmotic work is more accurately expressed by

$$nRT \left\{ g_b \frac{C_b - C_u}{C_u} + k_2 (\sqrt{C_b} - \sqrt{C_u}) - k_1 \ln \frac{C_b}{C_u} \right\},$$

when g_b , k_1 and k_2 are constants involving the osmotic activities of the ions in plasma and urine. We have used this formula, but under the conditions of our experiments the values given by the two formulae rarely differ by more than 10%.

Accurate computation of the total osmotic work requires a knowledge of the concentrations of every substance occurring in plasma and urine, but this involves what was to us an unmanageably large number of analyses in the types of experiment on diuresis described below, each sample of urine having a relatively small volume. The problem arises, therefore, as to the minimum number of analytical procedures which will yield a fair approximation to the total work. The solution adopted by Glaser *et al.* [1932] was to confine estimations to urea and chloride; that this would have given rather an incomplete picture of the total work under our experimental conditions is shown by the figures in Tables II-IV. We performed a fairly extensive series of estimations in preliminary experiments to discover a suitable compromise between convenient incompleteness and laborious accuracy in assessing the total work. In these experiments the following substances were estimated: (1) non-ionized: urea, creatinine, reducing substances; (2) anions: Cl , SO_4 , PO_4 , HCO_3 , lactate; (3) cations: NH_4 . Additionally (4) total electrolytes were estimated from the electrical conductivity, and (5) total molar concentrations were derived from vapour-pressure measurements. An example of the results of one of these experiments on the isolated kidney is given in Table I. It shows a discrepancy between the results of vapour-pressure measurement and the total obtained by the other estimations of 0.011 mol./l., about 3% of the total concentration. Bicarbonate was not estimated in this experiment, but from other experiments it would seem that practically the whole of the discrepancy of 0.0045 mol./l. between the anion concentrations determined by analysis and from conductivity measurements is due to bicarbonate.

TABLE I. Analysis of urine from isolated kidney showing the discrepancies (1) between vapour pressure measurement and substances estimated, and (2) between electrical conductivity and anions estimated

Substance	Molar concentration	Anion	Molar concentration
Urea	0.143	Chloride	0.0137
Creatinine	0.095	Sulphate	0.0003
Total electrolytes (conductivity)	0.041*	Phosphate	0.000
		Lactate	0.0035
Total (analyses)	0.279	Total (analyses)	0.0175
Total (calculated from vapour pressure)	0.290	Total (calculated from conductivity)	0.022
Difference	0.011	Difference	0.0045

* Corrected for apparent degrees of dissociation.

As a consequence of calculations of the partial osmotic works of the substances mentioned in these preliminary experiments, it became apparent that the greater proportion of the total osmotic work of the isolated kidney could be calculated from the concentrations of urea, creatinine and chloride in the serum and urine, and from the total electrolyte concentration (conductivity) in the urine. Sodium in the serum was taken as 1.3 times the serum chloride, and sodium in the urine was taken as the difference between cations estimated from the conductivity and the ammonia. Ammonia in the serum was estimated in a number of experiments and found to range from 0.5 to 2.5 mg./100 c.c. In most experiments it was assumed to be 1 mg./100 c.c. As can be seen in Tables II-IV the partial osmotic work due to ammonia is only a small fraction of the total work. Borsook & Winegarden [1931] calculated the work due to ammonia from the free energy changes in its formation from urea, attributing a considerable fraction of the total work to this reaction. Since urea is no longer regarded as the precursor of ammonia, and we preferred to avoid the confusion of chemical work on one substance only with the osmotic work on the other constituents of urine, we have included only the partial osmotic work done on ammonia as on the other substances.

This procedure of calculating the total osmotic work has been adopted in the experiments described below, and it is of interest to assess the order of the error involved in doing so. An upper limit to the work attributable to constituents which have not been estimated can be calculated for a typical urine with a creatinine urine/plasma concentration ratio of 20 if certain assumptions are made. The constituents may be considered in the following classes:

(1) *Undetermined substances which may be concentrated.* (a) Non-ionized: the difference in molar concentration between vapour pressure readings and the sum of substances estimated averaged 0.017 ± 0.005 (25) (cf. Table I). Assuming a U/P ratio as high as 10,

i.e. about that of urea, the partial osmotic work would be 15 ± 4 cal./l. (b) Anions: sulphate was excreted in so low a concentration that the partial work usually involved only a small fraction of a cal./l., and could be ignored. In about one-half our observations on pressure diuresis the difference between conductivity and chloride specified in 2 (b) below diminished with increase in urine flow, indicating that it represented in part substances which are concentrated in the urine. 1-2 cal./l. can be allowed for such substances.

(2) *Undetermined substances which are diluted.* (a) Non-ionized: reducing substances in the serum under our experimental conditions have usually been of the order of 0.005 *M*. Taking this as glucose completely reabsorbed by the kidney, the partial work would be 3 cal./l. (b) Anions: in thirty-eight experiments chloride accounted for about one-half the conductivity, the difference being 0.01 ± 0.0007 *M*. Bicarbonate accounts for about 0.004 *M* of this, and since in thirty-two analyses the CO_2 in arterial blood averaged 34.3 vol./100 c.c. or 0.014 *M* under our conditions, the serum not being more than this, the partial work on bicarbonate is about 3 cal./l. Part of the rest of the difference is due to lactate which often accounts for 1-2 cal./l., the whole group of anions other than chloride totalling 5-6 cal./l.

(3) *Cations.* Our calculation of work has supposed that the only cation in the urine other than ammonium is sodium. Isenberger & Tyler (1939) have, however, recently found that the concentration of potassium in urine from their form of heart-lung kidney preparation is of the same order as that of sodium. If this is so under our conditions, the concentration of potassium and the increased reabsorption of sodium involve extra work done which would rarely amount to 25 cal./l. In a urine in which electrolytes accounted for an unusually high proportion of the osmotic work, the partial osmotic works calculated by our usual procedure were urea 81, creatinine 88, chloride 23, ammonia 6, sodium 45, total 243 cal./l. Assuming, however, equal concentrations of sodium and potassium in the urine, the partial work on sodium would be increased to 60, and that done on potassium would be about 8, the other values remaining unchanged, total 266 cal./l., an increase of 23 cal./l.

At a creatinine U/P ratio of 20, the total osmotic work done by the isolated kidney calculated by our procedure is usually of the order of 300 cal./l. As can be seen from the preceding paragraph, a generous estimate of additional work done in concentrating and diluting those substances which we have ignored amounts to 25 cal./l. If in our experiments potassium was excreted in the same concentration as sodium, a further addition which might amount to 25 cal./l. would be due. This would suggest that the absolute values of total osmotic work calculated by our procedure are at most 14% lower than the true values, and that the error is likely to be between 5 and 15%. A scrutiny of the individual factors composing this error indicates that, during diuresis, these factors will change in the same way as the changes in our calculated work, and the errors in the variations in total osmotic work during diuresis, described below, are therefore likely to be smaller than the error in the absolute value of the work.

The absolute efficiency of the kidney has been calculated as the ratio of the total osmotic work to the energy corresponding with the oxygen consumption, the latter being converted to the appropriate units at the rate of 5 kcal./l. O_2 .

RESULTS

*The work done by the kidney and its efficiency
during pressure diuresis*

No more unambiguous example of "mechanical" diuresis could well be chosen than that due to an increase in arterial pressure applied to the isolated denervated kidney. We have observed the effects of pressure a number of times on each of eight kidneys, and have never failed to find a substantial and reversible increase of osmotic work associated with pressure diuresis.

Fig. 1 represents the results of one such experiment on the single pump-lung kidney preparation. It shows the characteristic effects of increase in arterial pressure in increasing the urine flow, creatinine clearance, osmotic work, oxygen consumption and blood flow, and it shows that these effects are of the same order of magnitude before and after raising the serum urea concentration from 0.032 to 0.186%. The effects are clearly reversible. The administration of urea itself produced relatively trivial changes in creatinine clearance, oxygen consumption and blood flow, but the urea diuresis, though a typical "tubular diuresis", was associated with an increase in osmotic work done which was not much greater than that due to the mechanical diuresis of the same order evoked by pressure change.

In twenty-three observations on pressure diuresis, the increase of osmotic work calculated for a doubling of urine flow averaged $38 \pm 3\%$.

The absolute efficiency of the kidney represented in Fig. 1 rises with arterial pressure, but rises more markedly as a consequence of adding urea, mainly because the change in oxygen consumption is much greater in pressure diuresis than in urea diuresis. In our series of isolated kidneys, the mean percentage increase in efficiency calculated as for a doubling of urine flow by increase in arterial pressure was $25 \pm 2\frac{1}{2}\%$.

A comparison of the efficiency of the kidney with that of other organs such as muscles suggests, as Borsook & Winegarden have pointed out, the calculation of the differential efficiency rather than the absolute efficiency. In muscular exercise the efficiency is computed in terms of the ratio of the increase in external work to the increase in oxygen usage, thus circumventing the difficulty that, owing to tone, the oxygen usage corresponding to a truly resting state of the muscles cannot be measured. The differential efficiency of the kidney can be calculated from the data depicted in Fig. 1, and ranges from 0.8 to 4.6%.

The increase of osmotic work done in diuresis depends on changes in concentration of a number of constituents of the urine, the individual constituents playing a different part according to the nature of the

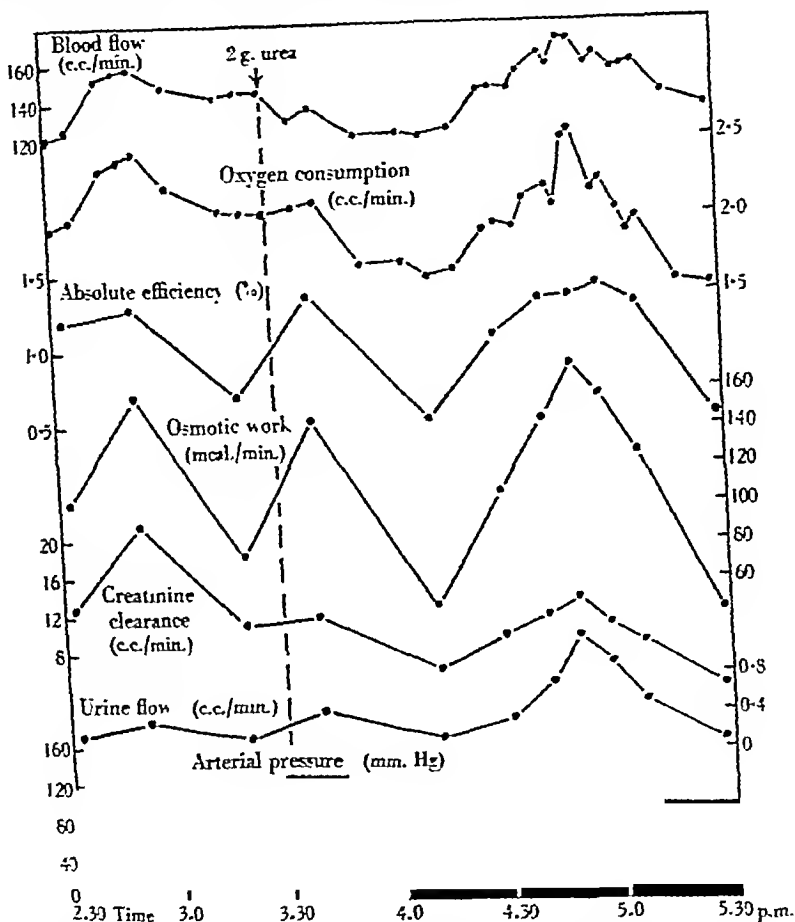


Fig. 1. Single pump-lung kidney preparation. The effects of arterial pressure on urine flow, creatinine clearance, osmotic work, efficiency, oxygen consumption and blood flow, before and after a dose of urea, showing the magnitude and reversibility of the phenomena. Kidney weight 30 g. Temperature 37°C .

diuresis. To illustrate the changes characteristic of pressure diuresis and the way in which the osmotic work has been calculated from them, details of an excerpt of an experiment on a single pump-lung kidney preparation are given in Table II. It will be seen that, in pressure diuresis, the concentrations of all the constituents of the urine tabulated move in the

TABLE II. The effects of rise and fall in arterial pressure;
an excerpt from an experiment on an isolated kidney

	Before	During	After
Arterial pressure (mm. Hg)	100	190	105
Urine flow (c.c./min.)	0.092	1.19	0.091
Creatinine			
Serum (%)	0.0225	0.024	0.034
Urine (%)	1.56	0.279	1.605
U/P ratio	69.5	12.4	47.5
Clearance (c.c./min.)	6.5	13.8	4.3
Partial osmotic work (mcal./min.)	26	27.5	23
Urea			
Serum (%)	0.181	0.193	0.230
Urine (%)	1.28	0.75	1.32
U/P ratio	7.1	3.9	5.75
Clearance (c.c./min.)	0.65	4.6	0.52
Partial osmotic work (mcal./min.)	13	50	11
Chloride			
Serum (% NaCl)	0.66	0.678	0.672
Urine (% NaCl)	0.014	0.044	0.014
U/P ratio	0.021	0.065	0.021
Clearance (c.c./min.)	0.002	0.077	0.002
Partial osmotic work (mcal./min.)	4	41.5	4
Conductivity			
Urine (molar NaCl)	0.021	0.016	0.018
Ammonia			
Urine (molar)	0.018	0.007	0.016
*Partial osmotic work (mcal./min.)	2.5	8	2.5
Sodium			
Serum (molar) = $1.3 \times$ molar chloride	0.147	0.151	0.15
Urine (molar) = conductivity - ammonia	0.003	0.009	0.002
Partial osmotic work (mcal./min.)	7	77	7
Total osmotic work (mcal./min.)	52.5	204	47.5

* Assuming a value of 1 mg. ammonia per 100 c.c. serum.

direction of the concentration in the serum. Nevertheless the work done in excretion is increased on account of the increase in the total amount of each of the constituents eliminated in unit time, as indicated by the increase in the clearances. The work done per litre of urine falls, but the work done per minute rises.

*The work done by the kidney and its efficiency
during urea diuresis*

Urea was chosen because it is generally regarded as a typical tubular diuretic. Its effects were studied in eight double pump-lung kidney preparations, one pump-lung circuit, the control circuit, being kept as

free as possible from added urea, and urea being added to the alternative test circuit in varying amounts.

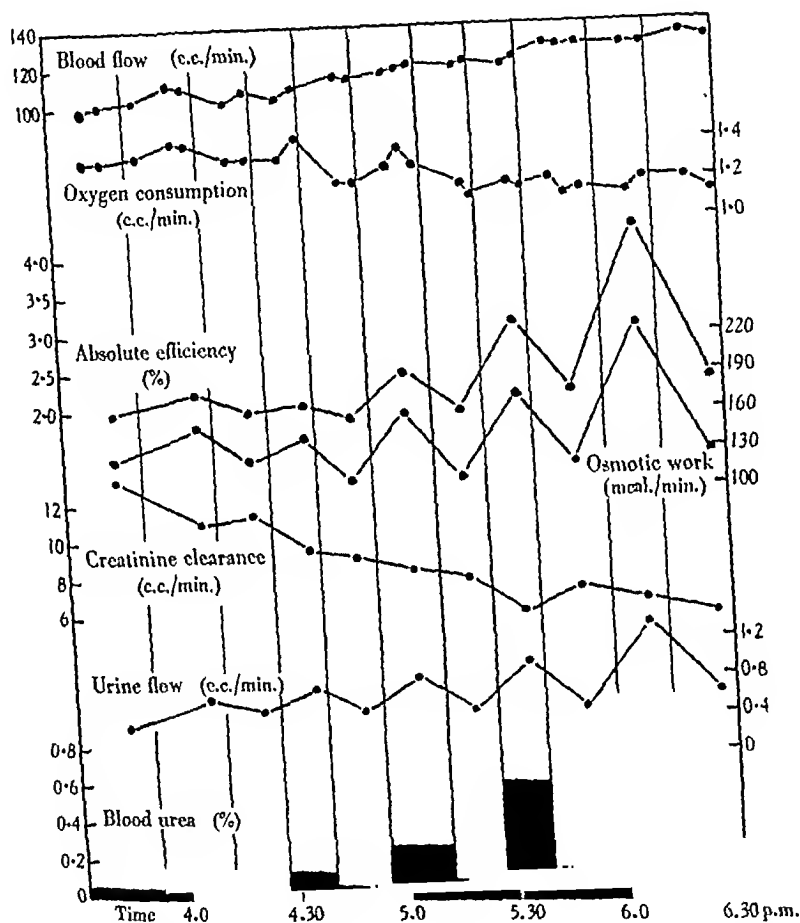


Fig. 2. Double pump-lung single kidney preparation. The effects of changes in the blood urea concentration at constant arterial pressure on the urine flow, creatinine clearance, osmotic work, efficiency, oxygen consumption and blood flow, showing the magnitudes with different urea concentrations and the reversibility of the phenomena. Kidney weight 23 g. Arterial pressure 125-130 mm. Hg. Temperature 37.5° C.

Fig. 2 represents one such experiment, showing progressive degrees of diuresis as the concentration of urea in the test circuit was raised. In contrast with the pressure diuresis shown in Fig. 1, the creatinine clearance remains substantially unaffected by changes in urine flow due

to urca, the small decrease in clearance sometimes accompanying diuresis being presumably due to the increase in intrarenal pressure. The slow progressive decline in clearance throughout the experiments is usual in kidneys perfused for long periods. The osmotic work, on the other hand, increases regularly with increase in urine flow due to urea as it does in pressure diuresis. In twenty-five observations on urea diuresis, the increase of osmotic work calculated for a doubling of urine flow averaged $63 \pm 7\%$.

Changes in the oxygen consumption are small compared with those in pressure diuresis, and there are correspondingly large changes in the absolute efficiency. The efficiency appears to increase progressively with increase in urea concentration in the blood, and reaches the remarkably high value of 4.3% at the highest concentration tested. There is no reason to suppose that it would not have risen even further at higher concentrations. In our series the average increase in efficiency during urea diuresis, calculated for a doubling in urine flow, was $59 \pm 9\%$.

It is difficult to attach any significance to differential efficiency in connexion with urca diuresis, for large increases of work may be associated with no detectable changes in oxygen consumption, as shown by the effects of the two highest concentrations of urca represented in Fig. 2. It seems clear that either the differential efficiency must approach 100% , and consequently involve changes in oxygen consumption so small that we have failed to detect them, or more probably that urea increases the absolute efficiency of the "resting metabolism" of the kidney.

Table III gives details of the changes affecting various constituents of the urine in part of a double pump-lung single kidney experiment when the kidney was switched from the control circuit to the high-urea circuit and back again. It will be seen that in urea diuresis, in contrast with pressure diuresis, there is no substantial change in the clearances of creatinine, urea and chloride, but that since the urea concentration in the serum has been deliberately raised, this implies a rise in the total output of urea in the urine in a given time, the total output of the other constituents being substantially unchanged. Although the U/P ratios fall, there is, therefore, an increase in the partial osmotic work attributable to urca, but a decrease in the work attributable to creatinine. The chloride in the urine is more dilute than that in the serum, and even more so during urea diuresis; this change accounts for the increase in the partial osmotic work attributable to this ion, although there is no great change in the total output of chloride.

TABLE III. The effects of rise and fall in serum urea concentration; an excerpt from an experiment on a double pump-lung single kidney preparation

	Before	During extra urea	After
Arterial pressure (mm. Hg)	125	125	125
Urine flow (c.c./min.)	0.40	0.76	0.42
Creatinine			
Serum (%)	0.0367	0.0263	0.039
Urine (%)	0.85	0.294	0.764
U/P ratio	23	11.2	19.6
Clearance (c.c./min.)	9.3	8.6	8.2
Partial osmotic work (mcal./min.)	40	18.5	35
Urea			
Serum (%)	0.08	0.286	0.094
Urine (%)	0.55	1.22	0.70
U/P ratio	9.2	4.3	7.5
Clearance (c.c./min.)	3.7	3.3	3.15
Partial osmotic work (mcal./min.)	30	63	34.5
Chloride			
Serum (% NaCl)	0.70	0.72	0.70
Urine (% NaCl)	0.012	0.004	0.012
U/P ratio	0.017	0.005	0.017
Clearance (c.c./min.)	0.007	0.005	0.007
Partial osmotic work (mcal./min.)	18.5	37.5	19
Conductivity			
Urine (molar NaCl)	0.0215	0.015	0.0105
Ammonia			
Urine (molar)	0.006	0.004	0.006
*Partial osmotic work (mcal./min.)	2	2	2
Sodium			
Serum (molar) = 1.3 × molar chloride	0.156	0.16	0.156
Urine (molar) = conductivity - ammonia	0.0155	0.011	0.0135
Partial osmotic work (mcal./min.)	17	38	18.5
Total osmotic work (mcal./min.)	107.5	159	109

* Assuming a value of 1 mg. ammonia per 100 c.c. serum.

The work done by the kidney during Ringer diuresis

Diuresis due to dilution of the blood with Ringer's solution has been generally regarded as a typically mechanical diuresis. This view of the mechanism of Ringer diuresis is almost certainly misleading in its application to the anaesthetized dog, for the diuresis is mainly due to inhibition of reabsorption of water, as we have shown elsewhere [1940]. In its application to the isolated kidney, however, the view is tenable so far as evidence derived from changes in the composition of urine is concerned, though the change in arterial pressure or ureter pressure needed to neutralize the diuresis is far greater than should correspond with the change in colloid osmotic pressure due to dilution of the serum proteins [Winton, 1937].

The double pump-lung single kidney experiment represented by Fig. 3 shows the changes in urine flow, creatinine clearance and osmotic work characteristic of progressive dilution of the blood. The dilution

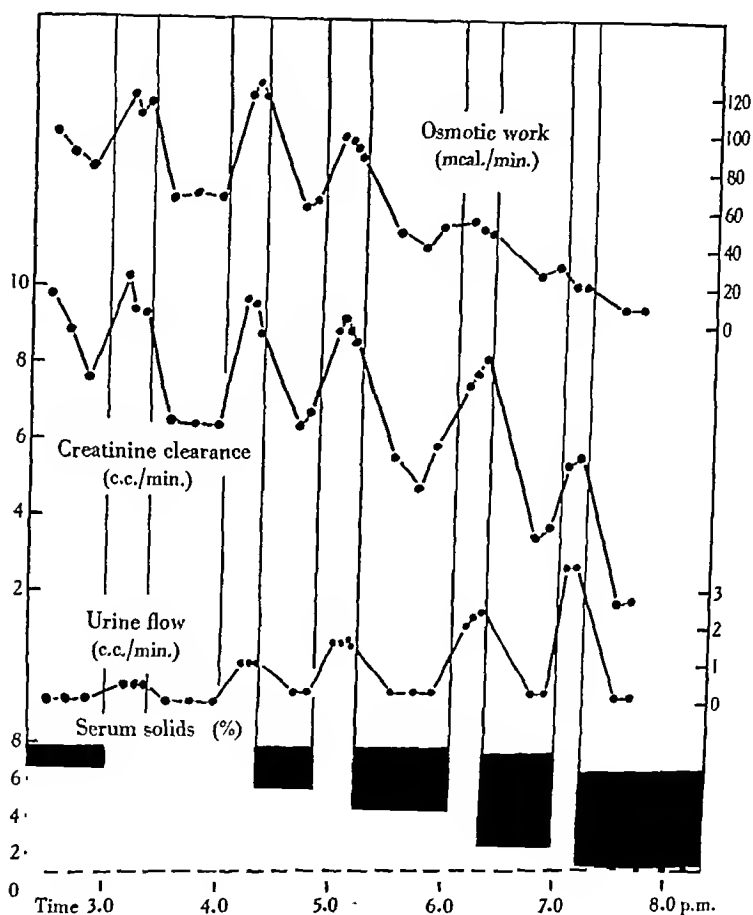


Fig. 3. Double pump-lung single kidney preparation. The effects of dilution of the blood with Ringer's solution on the urine flow, creatinine clearance and osmotic work. The broken white line indicates the level of solids in the serum other than proteins, so that the changes in serum solids measured from the broken line as a base line are an approximate measure of the changes in concentration of serum proteins. Kidney weight 15.5 g. Arterial pressure 110 mm. Hg. Temperature 37.5° C.

was produced by adding Ringer's solution to the venous reservoir in one circulation, urea, creatinine and glucose having been added to the Ringer's solution in amounts designed to minimize change.

concentration of these substances in the serum. The rise in both creatinine clearance and in osmotic work during Ringer diuresis correspond rather with pressure diuresis than with urea diuresis, in which no such increase in clearance occurs. In fourteen observations on Ringer diuresis, the increase of osmotic work calculated for a doubling of urine flow averaged $41 \pm 4\%$.

Little can at present be said about the change in efficiency during Ringer diuresis, because our method of measuring oxygen consumption

TABLE IV. The effects of rise and fall in serum proteins produced by dilution of the blood with modified Ringer's solution in a double pump-lung single kidney experiment

	Before	During Ringer addition		After
Serum solids (%)	7.4	6.35	6.4	7.4
Arterial pressure (mm. Hg)	110	110	110	110
Urine flow (c.c./min.)	0.27	1.0	0.99	0.31
Creatinine				
Serum (%)	0.0219	0.0212	0.0212	0.021
Urine (%)	0.575	0.27	0.252	0.416
U/P ratio	26	12.8	11.8	20
Clearance (c.c./min.)	7.3	12.7	11.8	6.3
Partial osmotic work (mcL/min.)	19	23	21	14
Urea				
Serum (%)	0.088	0.082	0.795	0.0875
Urine (%)	0.615	0.34	0.343	0.55
U/P ratio	7.0	4.2	4.3	6.3
Clearance (c.c./min.)	1.9	4.1	4.3	2.0
Partial osmotic work (mcL/min.)	18	21	22	16.5
Chloride				
Serum (%)	0.72	0.76	0.76	0.72
Urine (%)	0.120	0.122	0.118	0.144
U/P ratio	0.167	0.160	0.155	0.185
Clearance (c.c./min.)	0.045	0.16	0.154	0.063
Partial osmotic work (mcL/min.)	11.5	46.5	46	12
Conductivity				
Urine (molar NaCl)	0.0352	0.0358	0.0300	0.0363
Ammonia				
Urine (molar)	0.008	0.0045	0.0045	0.008
*Partial osmotic work (mcL/min.)	2.5	3.5	3.5	2.5
Sodium				
Serum (molar) = $1.3 \times$ molar chloride	0.16	0.173	0.169	0.16
Urine (molar) = conductivity - ammonia	0.0272	0.0313	0.0255	0.0283
Partial osmotic work (mcL/min.)	15	57	60	17
Total osmotic work (mcL/min.)	66	151	152.5	62

* Assuming a value of 1 mg. ammonia per 100 c.c. serum.

is not very suitable in circumstances involving large changes in concentration of the blood. If our impression that, for a given increase in urine flow produced by blood dilution, the change in oxygen consumption is smaller than that accompanying pressure diuresis is correct, Ringer diuresis would be accompanied by a greater increase in efficiency than the corresponding pressure diuresis.

In Table IV details of an experiment on Ringer diuresis are given which correspond with those for other forms of diuresis given in the previous tables. There is a general parallelism between Ringer and pressure diuresis in the respects depicted in the table, but a noteworthy feature of the Ringer diuresis is the large part which the increase in output of sodium chloride contributes to the total work done. The urine/plasma concentration ratio of chloride does not rise in Ringer diuresis, whereas in pressure diuresis it comes nearer to unity, and this in part neutralizes the increase in work which would be involved in the greater total amount of the ion excreted.

DISCUSSION

The change in osmotic work done during diuresis. The distinction in the current theory of renal secretion between the glomerular type of diuresis which is mechanical and the tubular type which involves secretory activity, has led many authors to suppose that the former is associated with no increase in oxygen consumption and with no increase in osmotic work done, whereas the latter is accompanied by an increase in both. In conflict with this supposition, a number of authors have shown that a rise in arterial pressure increases the oxygen consumption of the kidney, whereas the question as to whether tubular diuretics such as urea and sulphate do so is still in dispute. In the pump-lung kidney preparation of the dog, with which we are here concerned, there is always an increase of oxygen consumption with arterial pressure, but practically no such increase with urea [Kramer & Winton, 1939]. In further conflict with the theoretical supposition mentioned is the evidence now produced, showing that diuresis induced by an increase in arterial pressure or by dilution of serum proteins with Ringer's solution is accompanied by a substantial increase in the osmotic work done by the kidney. That this increase is, on the average, only a little less than that associated with a typical tubular diuresis due to urea is shown in Fig. 4. It is clear, therefore, that both the types of diuresis which have been regarded as mechanical and as tubular involve large changes in tubular activity,

and that the nomenclature implies an improper description of the mechanisms concerned.

Glaser *et al.* [1932] conclude from their observations on the urea and chloride excretion in anaesthetized dogs that there is no significant relation between the oxygen consumption and the osmotic work done.

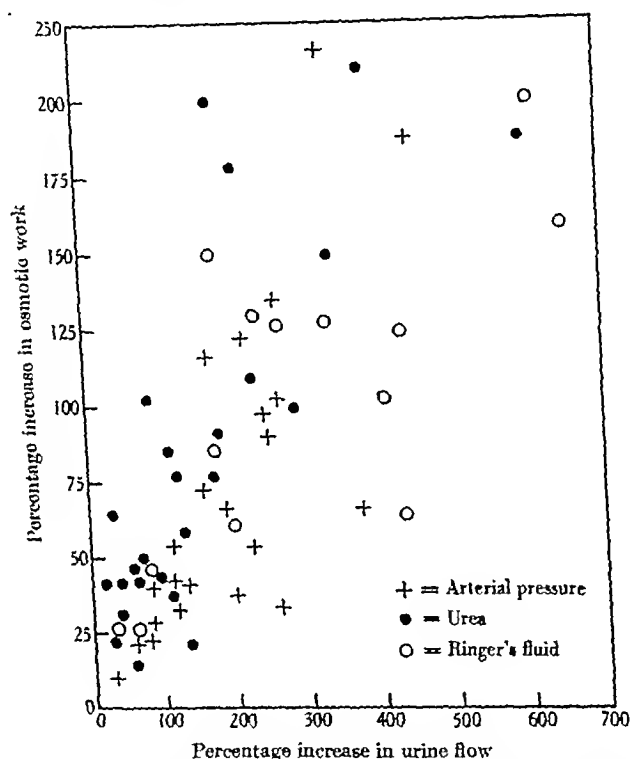


Fig. 4. The increase in osmotic work done during diuresis due to arterial pressure (+), Ringer's solution (○) and urea (●) showing the absence of a convincing distinction between "mechanical" and "tubular" diuresis. The average percentage increases in osmotic work for a 100% increase in urine flow are 38 ± 3 for arterial pressure, 41 ± 4 for Ringer's solution, and 63 ± 7 for urea.

We can confirm this conclusion in connexion with the isolated kidney. It would, however, be unsafe to assert that there is no relation between the metabolism of the kidney and the activity of the tubules, for the maintenance of an osmotic gradient across the tubule walls may involve activity of the cells and this would usually rise in circumstances, such as a low urine flow, in which the osmotic work done in the formation of urine falls. It would reach a maximum when the urine flow was abolished

by rise in ureter pressure or fall in arterial pressure. The metabolism of the kidney may, therefore, be related to its secretory activity in somewhat the same indirect way that the metabolism of muscle is related to its activity, though this activity may, as in isometric contraction, involve the performance of no external work.

The efficiency of the kidney. The most detailed previous discussion of the efficiency of the kidney is that of Borsook & Winegarden [1931]. These authors preferred a more complicated way of deriving the equation for the osmotic work done than that of von Rhorer [1905], which in essence we have employed, but the two equations are essentially the same. They applied their formula to a 24 hr. specimen of human urine, data concerning its constituents being derived from three sets of authors, and compared the results with determinations of oxygen consumption obtained during short periods by a number of different authors on the kidneys of anaesthetized or decerebrate animals and on isolated kidneys. This rather indirect calculation indicated an efficiency for the kidney of about 1%.

In our observations on the isolated kidney, the oxygen consumption was continuously recorded while the samples of urine were being collected, and reasons have been given for supposing that our calculated value of the osmotic work done was about 90% of the true value. Efficiencies in our experiments varied from zero to about 5%, the highest values being obtained under the influence of high concentrations of urea. Still higher values could, in all probability, have been obtained if the serum urea concentration had been raised further. We can confirm Borsook & Winegarden's estimate of the efficiency as 1% as a fairly usual value when urine is secreted at the normal "resting" rate.

Borsook & Winegarden distinguish the absolute efficiency, i.e. the ratio of the total osmotic work done to the total metabolism, which is discussed above, from the differential efficiency, i.e. the ratio of the changes in these quantities. They regard the latter as the more significant, and estimate it by comparing the change in oxygen consumption of human subjects due to drinking a solution of urea with the changes in urea and chloride excretion. They conclude that the differential efficiency of the kidney is of the same low order as the absolute efficiency. Van Slyke, Rhoads, Hiller & Alving [1934], however, found no systematic change in the oxygen consumption of the kidney of conscious dogs as a result of urica administration, and the increase in metabolic rate of the human subjects mentioned above was presumably extrarenal in origin.

Simultaneous observations on the change in osmotic work done as a result of urea administration and on the change in oxygen consumption of the isolated kidney make it difficult to attach any meaning to the differential efficiency, for while there is always a substantial increase in work done, there is usually little or no increase in oxygen consumption.

SUMMARY

1. Estimations of creatinine, urea and chloride in serum and urine, and of the ammonia content and electrical conductivity of the urine, have been shown to be adequate to yield a fair value of the total osmotic work done by the isolated kidney of the dog, probably 5-15 % lower than the true value.

2. In the pump-lung kidney preparation, a rise in arterial pressure produces diuresis accompanied by increases in osmotic work, in creatinine clearance, in oxygen consumption, and in efficiency (Fig. 1). For a doubling of urine flow the increase in osmotic work is 38 ± 3 % and that in efficiency 25 ± 2.5 %.

3. In urea diuresis there is a greater increase in osmotic work with little change in creatinine clearance or oxygen consumption, the efficiency being increased (Fig. 2). For a doubling of urine flow the increase in osmotic work is 63 ± 7 % and that in efficiency 59 ± 9 %.

4. In Ringer diuresis in the isolated kidney there are increases in the osmotic work and in the creatinine clearance (Fig. 3). For a doubling of urine flow the work is increased 41 ± 4 %.

5. "Mechanical" and "tubular" types of diuresis cannot safely be distinguished by the differences in the increase in osmotic work they involve (Fig. 4), the increase in work in pressure diuresis being only just significantly less than that in urea diuresis.

6. The efficiency of the isolated kidney calculated as the ratio of the total osmotic work to the oxygen consumption, simultaneously determined, is often about 1 %. It falls to zero when the urine flow is abolished, increases during all the forms of diuresis studied, and reaches high values, e.g. 5 %, during extreme urea diuresis. For a doubling in urine flow the increase in efficiency is 25 ± 2.5 % in pressure diuresis and 59 ± 9 % in urea diuresis.

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THE PRODUCTION OF EXOPHTHALMOS IN THE DOG BY ACETYLCHOLINE

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STIMULATION of the cervical sympathetic trunk in the dog and other animals usually produces exophthalmos. Working with cats Bacq & Frédéricq [1934] found in about half the animals that some of the nerve fibres of the nictitating membrane were cholinergic and not adrenergic. Preliminary experiments by the present writer showed that the injection of acetylcholine into the lingual artery of the anaesthetized dog was usually followed by proptosis. Proptosis is used here as a synonym for "forward movement of the eyeball" and is not intended to describe the means by which the movement is produced. Four series of experiments were then planned in order to test possible sites of action by the drug: (1) the origin in the superior cervical ganglion of postganglionic fibres innervating Müller's orbital membrane (Müller's orbital muscle), (2) cholinergic nerve endings in the orbital membrane of fibres originating in the ganglion or elsewhere, (3) the muscle fibres of the membrane itself, and (4) arterioles, capillaries and veins behind the eyeball which might be dilated by direct action of the acetylcholine and so might cause protrusion of the eyeball. In the first series injections of acetylcholine, with or without eserine, were made into the lingual artery before and after acute removal of the superior cervical sympathetic ganglion. Proptosis after removal of the ganglion would show the existence of a response at one or more of the sites 2, 3 and 4. In the second series the injections were made 3 or 4 weeks after the removal of the ganglion and hence, as it is believed, after degeneration of the postganglionic fibres which supply the orbital membrane. Proptosis in this series would show a response at either site 3 or 4 or at both. In the third series, isolated heads, deprived acutely of one superior cervical ganglion, were perfused with a constant inflow of blood. The constant inflow would ensure that any relaxation

of the vessels produced by acetylcholine would not be followed by an increased blood flow. Hence the veins would be able to deal with the blood supply, and no protrusion of the globe would be produced unless the drug acted directly on the muscle fibres of the membrane. The fact that injections of acetylcholine did not necessarily alter the outflow of blood was confirmed by experiments. The plan of the experiments is shown diagrammatically in Fig. 1.

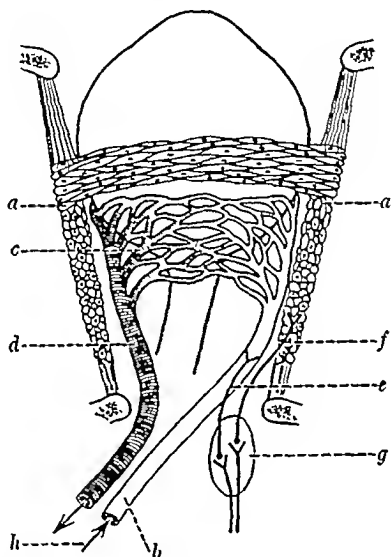


Fig. 1. Diagram of nervous and vascular factors of exophthalmos in the dog. In the normal orbit the following structures are intact: (a) Müller's orbital membrane, partly cut away to show the vessels inside it, (b) artery of supply to the orbit, (c) capillaries, (d) veins, (e) vasomotor nerves, (f) postganglionic nerves to the orbital membrane, (g) superior cervical ganglion, and (h) central control of the blood supply to the orbit. In experiments of series 1, (g) was removed. In those of series 2, (e), (f), and (g) were all absent. In those of series 3, (g) was removed and the supply of blood was no longer controlled by the animal's central nervous system.

On the basis of phenomena noted by a colleague during experiments on exophthalmos in dogs, the colleague had suggested the possibility of fibres passing up to the orbits along the vertebral arteries. This possibility was important for the interpretation of the results in series 1 and 2. Though it was supported by few, if any, of the results obtained in series 3, it was also tested by direct experiments in series 4. In these the stellate ganglion was stimulated electrically and the response of the eyeball was measured before and after cutting the cervical sympathetic nerve trunk.

METHODS

The animals were anaesthetized with ether followed by chloralose. In the first series of experiments a cannula was placed in the lingual artery so that injections could be made through it into the external carotid artery which supplies the orbital cavity in the dog. The superior cervical ganglion was exposed, and was made ready for removal at a later stage in the experiment. The dog's head was fixed and movements of the eyeball were recorded by means of a lever previously described [Brunton, 1935]. It was thus possible to record the effects of (1) maximal stimulation of the cervical sympathetic nerve trunk, and of (2) injections of various substances into the artery both before and after the removal of the superior cervical ganglion. The injected substances were "washed in" with subsequent injections of saline. The response to nerve stimulation gave a rough standard with which to compare the response to injections. In all the experiments on which this communication is based, subsequent examinations showed that the ganglion had been completely removed.

The second series of experiments differed chiefly from the first series in the removal three or more weeks before the final experiment of one superior cervical sympathetic ganglion, and the assumption that the postganglionic fibres degenerated during the interval. The adrenal veins were ligated and the injections were made into the femoral vein. The third series of experiments, however, makes this series of little importance, since even the *acutely* denervated orbital membrane did not respond to acetylcholine when the volume of blood supplied to the orbit in unit time was kept constant.

In the third series, dogs' heads were completely isolated and were perfused, through a double cannula placed in the common carotid arteries, with oxygenated defibrinated blood from the dog itself or from a second dog. The cannula had a side tube leading to a manometer which recorded the pressure of blood entering the carotid arteries ("cannula pressure"). The perfusion circuit was completed by a Dale-Schuster pump, a Hooker oxygenator, cannulae in the external jugular veins (or alternatively a collecting funnel beneath the head) and the necessary connexions. Except in the first two experiments glucose was added half-hourly to the perfusing blood, and the pressure in the arterial cannulae was kept between 70 and 120 mm. Hg. In all the experiments the delivery of the pump was carefully watched. Except in certain experiments where it was specially desired to test the effect of increasing or decreasing the

blood supply, the delivery was constant during the periods when observations were being made on the eyes. This constancy of supply prevented relaxation of vessels being followed by their distention with blood, except in two experiments which are discussed below.

The cervical sympathetic nerves were identified by the effect of electrical stimulation just caudal to the superior cervical ganglion, which was dissected out, usually on the right side, during the dissection of the neck for the ligation of its muscles and the vertebral arteries. The ganglion was removed before the circulation was interrupted between head and body. (In the first two experiments of this series both ganglia were left intact, and in the first the perfusion pressure was only about 35 mm. Hg. The results obtained in these experiments are included with results on the "innervated" orbits in the other experiments.)

Movement of the globes was at first recorded by removing the eyelids and fitting the margin of each orbit with a funnel oncometer which was connected to a Brodie's bellows. The movements of each bellows were recorded on smoked paper by its writing point. This enabled the time relations of the eyeball to be studied. Later an exophthalmometer was designed. Its use saved about 30 min. formerly occupied in fitting the oncometers, and it enabled the forward or backward movements of the eyeball to be measured in absolute units with a greater accuracy than 0.3 mm.

Criteria of vitality of the preparations included a considerable difference of colour in the blood entering and leaving them, an exophthalmic response to stimulation of the cervical sympathetic nerve trunk on the side with an intact ganglion and a response to adrenaline on the side deprived of its ganglion. These criteria were sought whenever the globe failed to respond to acetylcholine.

In the fourth series (5 dogs) the stellate ganglion was exposed and stimulated electrically before and after cutting the vagosympathetic nerve trunk at about the level of the thyroid cartilage. Movements of the eyeball were recorded as in series 1 and 2.

RESULTS

(1) *Acetylcholine after acute superior cervical ganglionectomy*

In nine dogs stimulation of the cervical sympathetic nerve trunk produced a maximal proptosis of about 6 mm. In two dogs, even before ganglionectomy, repeated injections of acetylcholine in doses which

varied from 1.0 to 50.0 μg . had no effect. Usually a maximal effect was obtained by doses of 5 or 10 μg . In the other seven dogs acetylcholine produced a proptosis of from 1.0 to 5.5 mm. (Fig. 2). The proptosis usually preceded any change of arterial pressure (Fig. 3). The degree of proptosis was little altered by ganglionectomy, hence it was concluded that the chief site of action was not the superior cervical ganglion.

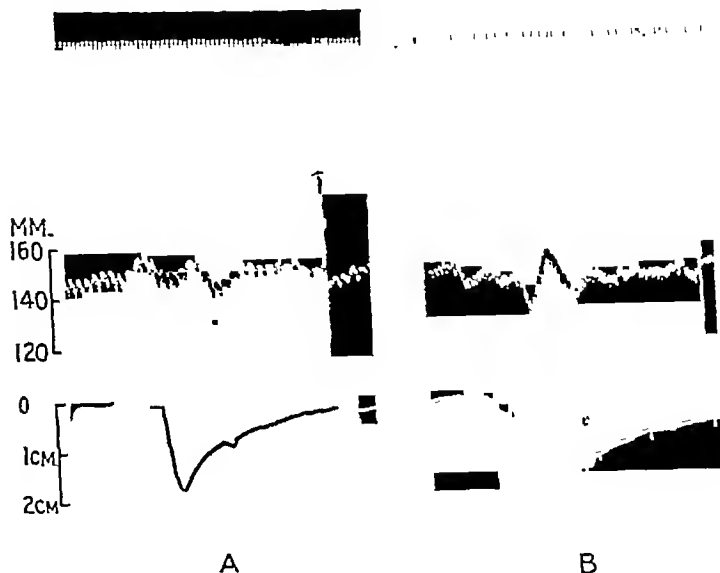


Fig. 2. Proptosis due to acetylcholine. Records from above downwards show time in seconds, blood pressure in the femoral artery and movements of the eyeball. Descent of the eyeball line shows proptosis. The vertical lines near the end of each record are alignment marks. A shows response of the eyeball to stimulation of the cervical sympathetic nerve before ganglionectomy. B shows the response to interarterial injection of 10 μg . acetylcholine after ganglionectomy. The absolute amounts of proptosis in A and B were 4.9 and 5.5 mm. respectively.

(2) *Acetylcholine after chronic superior cervical ganglionectomy*

Injections of from 1.0 to 50.0 μg . acetylcholine into the femoral vein produced, on the "denervated" side, an exophthalmic response which was about one-third of the maximal response to nerve stimulation recorded on the innervated (normal) side by Brodie's bellows. It was concluded that the drug could produce exophthalmos in the absence

of sympathetic nerve endings in Müller's orbital membrane and, therefore, acted on the membrane itself, on the orbital vessels or on both these tissues.

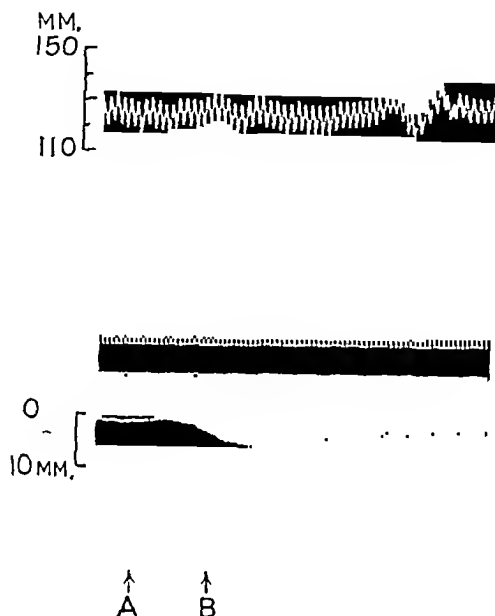


Fig. 3. Response of the eyeball to acetylcholine preceding change in femoral artery pressure. Records from above downwards show femoral artery pressure, time in seconds and movements of the eyeball. From A to B 8 μ g. acetylcholine were injected. Proptosis began after 10 sec. and reached a maximum of 1.4 mm. absolute. The femoral artery pressure only altered after about 60 sec.

(3) *Head perfusions with unilateral acute superior cervical ganglionectomy*

The doses of acetylcholine varied from 10 to 200 μ g., the usual doses being 50 or 100 μ g. The globe on the side from which the ganglion had been removed only altered its position in three out of twenty-seven experiments. These three responses are considered below. The response varied on the side with an intact ganglion. One dose of 500 μ g. produced 2.0 mm. protrusion (Fig. 4); seven doses of 50 or 100 μ g. produced 0.5–1.0 mm. protrusion; two doses of 100 μ g. produced doubtful protrusion of less than 0.5 mm. Two doses of 50 μ g. produced 0.5–1.0 mm. retrocession; one dose of 50 μ g. produced more than 2.0 mm. retrocession. Nineteen doses, varying from 10 to 500 μ g., produced no change. A few

other doses, given after the perfusion had proceeded for a long time, produced no change, but are not included owing to doubt as to the responsiveness of the orbital membrane.

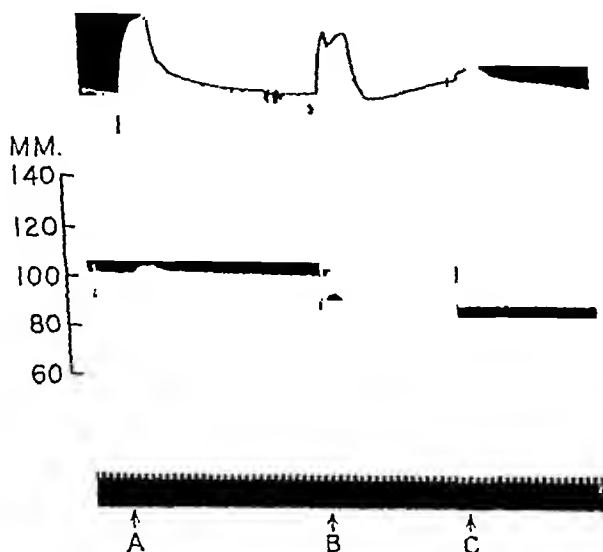


Fig. 4. Effect of nervous stimulation and of acetylcholine after eserine on a perfused head preparation with intact superior cervical ganglion. Records from above downwards show movements of the eyeball recorded by a Brodie's bellows, perfusion pressure and time in seconds. At A, maximum nervous stimulation. At B and C, two consecutive injections of 500 μ g. acetylcholine. After the second of these no further response was obtainable. In Figs. 4 and 5, ascent of the eyeball line shows proptosis.

(4) *Stimulation of the stellate ganglion after section of the cervical vagosympathetic nerve trunk*

After cutting the nerve trunk, stimulation of the stellate ganglion had no effect on the position of the eyeball, the pupil or the nictitating membrane. In these animals, therefore, no fibres passed through the stellate ganglion or were relayed in it on their way to Müller's orbital membrane except those fibres which passed to the membrane by the cervical sympathetic nerve trunk.

DISCUSSION

In the light of the results just described, it is possible to say whether or no acetylcholine acts at each of the following sites: (1) the origin in the superior cervical ganglion of the postganglionic fibres of Müller's orbital membrane, (2) cholinergic nerve endings in the membrane itself of fibres originating in the superior cervical ganglion or elsewhere, (3) the muscle fibres of the membrane, and (4) the vessels of the orbital cavity. The results of series 1 and 2 showed that acetylcholine altered the position of the globe in the absence of the postganglionic nerves which had their origin in the superior cervical ganglion. In series 3 and 4 were investigated some possible explanations of this exophthalmos: (a) stimulation of cholinergic nerve endings in Müller's orbital membrane which would have an origin outside the superior cervical sympathetic ganglion, (b) direct stimulation of the membrane itself, and (c) dilatation of orbital vessels with relative insufficiency of venous outflow so that protrusion resulted from vascular engorgement.

In the perfused heads of series 3 acetylcholine failed in twenty-four out of twenty-seven cases to produce any effect on the eyeball on the side from which the superior cervical ganglion had been removed. Two of the positive responses occurred in a preparation in which, unfortunately, the completeness of removal of the ganglion could not be investigated after the experiment. Some fragment of the ganglion may have been left with an intact blood supply and may have received some acetylcholine. This is improbable, even though the doses which produced these exceptional positive results produced exophthalmos of exactly the same degree on the normally innervated eye. Vascular engorgement is believed to have been the mechanism in these two cases as the doses of acetylcholine were large and as a temporary reduction took place in the outflow from the orbits after the injections. Subsequent experiments, not included in this article, have confirmed this belief. A third possible explanation is that, in this particular dog, fibres originating in the sphenopalatine or some other ganglion (but not in the superior cervical ganglion) supplied part or all of Müller's orbital membrane, and that these fibres were stimulated at their origin by the acetylcholine. It will be remembered that, in Müller's original communication [Müller, 1858], it was stated that the orbital membrane was supplied with nerves which could "be traced anatomically in part to the sphenopalatine ganglion". The present writer is unable to decide whether this description was applied to the membrane in the lower animals or to the muscle in

the human orbit. If the nerve supply to the orbital membrane of dogs always originated in the sphenopalatine ganglion, the membrane would have responded in all the head perfusion experiments. If the preganglionic fibres had originated in or passed through the stellate ganglion, stimulation of the stellate ganglion would have produced responses in the experiments of series 4. The conclusion is drawn that in few, if any, cases does the nerve impulse reach Müller's orbital membrane in the dog by any other path than that from the superior cervical ganglion. The question of the innervation of the muscle in man is left open by these experiments, but in man Müller's orbital muscle cannot produce exophthalmos [Whitnall, 1932; Brunton, 1938]. A possible difference of route for sympathetic impulses in the orbits of man and the dog may be considered in connexion with the different main artery of supply to the orbit: the internal carotid in the case of man and the external carotid in the case of the dog. The work of Pochin [1939] should also be consulted.

The third positive result produced by acetylcholine on a "ganglionectomized" orbital membrane cannot be explained on any of the three suppositions suggested in the last paragraph, since, in response to two preceding and three subsequent injections, no exophthalmos occurred. This positive case is believed to be due to faulty technique and the negative responses should probably be reported as twenty-five out of twenty-seven. The conclusion is drawn that in doses of from 10 to 500 μ g. acetylcholine does not act directly on Müller's orbital membrane in the dog.

In order to decide whether acetylcholine causes proptosis by its action on the blood vessels, it may be recalled that, after superior cervical ganglionectomy, it produced proptosis in the animals with intact vascular reflexes and variable blood supply to the head, but that it rarely if ever produced proptosis in the head preparations with a constant blood supply when the superior cervical ganglion had been removed. The conclusion is drawn that, in the intact dog, acetylcholine produces protrusion of the eyeball by vascular engorgement as well as by stimulation of the superior cervical ganglion.

In support of this conclusion three facts may be mentioned. (i) When acetylcholine was injected into the head preparations, dilatation of vessels was sometimes shown by a fall of about 10 mm. Hg in the cannula pressure, and with constant pump supply the outflow from the head sometimes decreased slightly. (ii) On the other hand, if the pump supply of blood was purposely increased or diminished, especially if the initial

cannula pressure was low, exophthalmos or enophthalmos was produced. Fig. 5 shows such effects recorded by a Brodie's bellows. Table I shows absolute amounts of exophthalmos produced in innervated and denervated orbits by intentional increase of blood supply. Only one response to a decreased supply is shown, as there was a variable lag in the return of the eyeball after protrusion, so that it was often gently pressed back in order to save time.

(iii) As shown in Table II exophthalmos could be produced by intentional obstruction of the external jugular veins. This reduced the outflow from the head (as shown in column 1), while the pump supply remained unchanged. Similar effects were obtained on cats by Whitnall & Beattie [1933] when the circulation in the jugular veins was obstructed. No measurements of the degree of exophthalmos were given. Their results and those given in Fig. 5 and Tables I and II emphasize the importance of circulatory changes in the orbit of the dog. So does the enophthalmos which follows death or fall of blood pressure in the carotid arteries.

A complete interpretation of the results on head preparations with constant blood supply but intact superior cervical ganglia will not be attempted in this paper. The relation of the response to the size of the dose of acetylcholine, to the administration of eserine, to the initial perfusion pressure and to changes in it and in the venous outflow following the injections are all under consideration. So, too, are the effects of anaesthesia [Essex & Corwin, 1937].



Fig. 5. Response of the eyeball in an isolated head preparation to experimental changes of perfusion pressure caused by alteration of the stroke of the pump. Records as in Fig. 4.

TABLE I

Experimental alteration cannula P mm. Hg	Resulting change of outflow c.c./min.	Resulting change of innervated eye mm.	Resulting change of denervated eye mm.
104-158	?	0.5 Ex.	1.0 Ex.
102-160	32-94	0.5 Ex.	1.0 Ex.
130-160	51-83	2.0 Ex.	2.0 Ex.
86-65	94-60	1.0 En.	0.5 En.

TABLE II

Experimental alteration of outflow c.c./min.	Resulting change cannula P mm. Hg	Resulting change of innervated eye mm.	Resulting change of denervated eye mm.
100-83	85-105	1.0 Ex.	2.0 Ex.
200-168	115-135	1.0 Ex.	2.0 Ex.
?	105-112	2.0 Ex.	1.0 Ex.

SUMMARY

Injections of acetylcholine were made under chloralose anaesthesia into dogs from which one superior cervical sympathetic ganglion had been removed either at the time of operation (series 1) or sufficiently long beforehand to allow degeneration of the nerves which supplied Müller's orbital membrane (series 2). The injections produced about the same amount of exophthalmos on the denervated side as on the side where the ganglion and its nerves were intact. Hence they affected either the orbital membrane, the orbital vessels or both these tissues.

By perfusing isolated dogs' heads, it was possible to provide a constant blood supply to the orbits. Under this condition acetylcholine produced exophthalmos in only two cases out of twenty-seven in which the ganglion had been removed. This fact and other evidence leads to the conclusion that the main action of acetylcholine in the intact animal is a relaxation of blood vessels. The relaxed vessels are then distended with blood and push the eyeball forwards.

I am grateful to Prof. Roaf for his encouragement and continued interest in this work. I am indebted to the Medical Research Council for permission to carry out the earlier experiments of series 3 at the National Institute for Medical Research, Hampstead. I wish to thank the Council and Sir Henry Dale, the Director of the Institute, for the opportunity and assistance there afforded me.

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EFFECTS OF INCREASED METABOLISM ON KETOSIS OF DEPANCREATIZED DOGS

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RECENT research on the modification of experimental diabetes by removal of the pituitary [Houssay, 1937] or adrenals [Long, 1937], and by prolonged inanition [Barker, Chambers & Dann, 1937] has emphasized the necessity for careful evaluation of the oxidation of carbohydrate in respect to other metabolic changes in the body. One of these is the relationship of the elimination of ketone bodies to the oxidation of carbohydrate. Although some investigators question the interpretation of the respiratory quotient (R.Q.), when adequately controlled it affords the most reliable method of determining the fuels oxidized by the whole body. Studies in this laboratory of normal and depancreatized animals after hypophysectomy or adrenalectomy have shown a close correlation between the R.Q. and the intake of carbohydrate or the amount of glucose excreted over extended periods of time [Chambers, 1938]. No demonstrable oxidation of administered glucose has been found in the depancreatized dog in the early stages of inanition, and the ingested sugar is quantitatively excreted. On the basis of this evidence, there is no reason to assume that there is oxidation of glucose, either endogenous or exogenous. This animal would, therefore, rely almost entirely upon fat and fatty acid residues for its energy supply, and should excrete a corresponding amount of ketone bodies, according to the concept of a definite relation between the amount of carbohydrate and of ketogenic materials which must be metabolized simultaneously in order to prevent ketone formation from the latter. For each calorie of heat produced by a depancreatized dog, there should be excreted 0.36/8.5 g. ketones,

computed from a diabetic heat value of 8.5 calories¹ and an acetone body yield of 0.36 g. per g. of fat oxidized [Magnus-Levy, 1908]. We have calculated the ketosis to be expected on this basis during seventy-four basal periods of at least 3 hr. each, obtained on seventeen depancreatized dogs (Table I). In no case was there found even approximate agreement between theoretical and actual ketonuria—the highest amount obtained was only 25% of that expected, and the others were about 10%.

TABLE I. Differences between actual and calculated amounts of acetone bodies excreted by depancreatized dogs

Dog no.	No. of periods	R.Q.	Heat cal./hr.	Urine			Acetone bodies calculated mg./hr.	
				N mg./hr.	D : N	Acetone bodies mg./hr.	From fat	From protein
B 1	2	0.71	34.91	389	3.8	130	1480	226
B 3	3	0.73	22.63	179	2.9	150	953	104
B 4	1	0.71	14.74	210	3.3	92	623	122
B 5	13	0.72	16.55	135	2.3	53	700	78
B 6	1	0.71	28.39	332	4.3	315	1200	193
B 11	7	0.71	20.07	176	2.4	36	649	102
B 13	3	0.70	28.40	293	3.1	133	1203	173
B 14	2	0.72	27.86	379	3.1	267	1177	220
B 15	1	0.71	13.46	272	3.4	118	569	158
B 16	6	0.72	26.44	199	2.8	73	1118	115
B 17	4	0.73	18.07	233	2.2	17	765	138
B 18	6	0.71	23.78	181	1.7	32	1004	105
B 20	4	0.72	18.66	144	3.8	93	789	84
B 24	12	0.72	23.07	210	3.8	79	976	122
B 31	1	0.72	34.15	250	2.3	25	1445	145
B 35	5	0.73	19.99	158	2.5	20	906	92
B 39	3	0.71	26.73	201	3.7	324	1131	117

It should be pointed out that inclusion of the ketones derived from the breakdown of protein would raise the total calculated value still further. If leucine, phenylalanine, and tyrosine are considered the only ketogenic amino-acids [Shaffer, 1921], from the data on amino-acid content of muscle protein, it can be computed that about 58 mg. ketone bodies (in terms of acetone) should be formed per 100 mg. protein nitrogen. In twelve of the seventeen animals listed in Table I, the actual acetone excretion was less than that calculated from protein alone. These calculations shed no light, of course, on the actual source of the acetone bodies in respect to fat and protein.

The inconsistency between actual and calculated excretions of acetone bodies during the periods of basal metabolism suggests that a method

¹ If the glycerol heat alone is subtracted from the caloric value of 9.3 for fat, 8.76 is obtained. If three moles of β -hydroxy butyric acid are also deducted, the heat value falls to 7.44 [Chambers, Himwich & Kennard, 1935]. Since some acetone is excreted, but not the theoretical amount, the factor of 8.5 was selected for use in the present calculations.

of increasing the metabolism of fat might be used to better advantage to test the quantitative production of acetone from fat in the depancreatized dog. Experiments consisting of the administration of fats by mouth are of doubtful value, due to impaired absorption. Even if intravenous injection is resorted to, the exogenous fat may be partially substituted for endogenous, with very little change in the total amount oxidized. To obviate such difficulties of interpretation, it was decided to accelerate the oxidation of endogenous fat by increasing the metabolic rate of depancreatized animals. The increased oxidation of fat should produce an equivalent increase in the ketosis, if the quantitative production theory discussed before holds true. In the preliminary report [Barker, 1936], we compared three means of heightening the oxidation of fat—exercise, injection of dinitrophenol, and administration of desiccated thyroid substance—and this paper will present in more detail the results obtained from exercise and dinitrophenol.

There appear to be no published reports on the acetone excretion of depancreatized animals as affected by exercise or dinitrophenol (D.N.P.). Studies in the literature concerning the effects of exercise on ketosis can be divided into three general types of experiments: those on phlorrhizinated dogs, on diabetic patients, and on humans or animals subsisting on low-carbohydrate diets [cf. Richardson & Levine, 1925]. There is wide variation in the results, as might be expected from such a diversity of experimental subjects. Furthermore, in only a few of the reports was any attempt made to learn the nature of the foodstuffs oxidized during the periods of increased metabolism. Since the subjects were quite able to burn carbohydrate, the quantitative estimation of ketonurics in these cases must be of doubtful importance.

METHODS

An open-circuit form of respiration apparatus was employed, the air analyses being done with a Carpenter-Haldane gas analyser [Carpenter, 1933]. The accuracy of the method is attested by the constancy of the customary alcohol and acetone checks: seventeen alcohol R.Q.'s averaged 0.669 ± 0.004 , and five acetone checks averaged 0.751 ± 0.002 . Continuous samples of the air leaving the animal chamber were withdrawn by means of a mechanical device in order to insure a truly representative aliquot for the entire duration of each respiration period [Barker & Smyth, 1938]. A wet meter was used to measure the ventilation, enabling the collection of expired air acetone in a bisulphite solution. The importance of collecting the expired air acetone is well illustrated in Table II,

TABLE II. "Blowing-off" of acetone in depancreatized dogs submitted to exercise or given dinitrophenol

Experiment	Dog. no.	Basal acetone mg./hr.		Experimental acetone mg./hr.	
		Air	Urine	Air	Urine
Exercise	B 5	4	62	10	74
	B 3	35	165	131	139
	B 11	4	15	43	32
	B 13	16	109	32	101
Dinitrophenol	B 1	13	119	39	96
	B 6	53	262	178	94
	B 16	24	221	35	191
	B 18	11	18	25	8

from which it is seen that exercise and dinitrophenol both caused temporary "blowing off" of acetone.

Adult mongrel female dogs were depancreatized in one stage, and maintained for several days after the operation on a liberal diet coupled with insulin therapy.¹ Total absence of residual pancreatic tissue was verified in each case at autopsy. After food and insulin were withheld, at least 3 days were allowed to elapse in order to rid the body of all possible stores of insulin. It has been shown that, for at least 2 weeks after this preliminary period, or until the weight loss has reached 45 %, there is no oxidation of glucose ingested by the depancreatized dog [Barker *et al.* 1937]. Six of the animals used in the present study were also used for glucose experiments. In the exercise series, resting metabolism was determined in hourly periods for 3 or 4 hr., after which the animal was run on a motor-driven treadmill at the rate of about 6 km. per hr. It was found possible to exercise depancreatized dogs only as long as 45-55 min. without loss of urine; a recovery period of 3 hr. was obtained following the exercise. The detailed data obtained from a typical experiment are shown in Table III.

When D.N.P. was used, 1-4 mg. of the drug per kg. were dissolved in warm 1 % NaHCO₃ solution, sterilized, and injected intraperitoneally after the basal metabolism had been determined. The animals were then replaced in the chamber and the respiratory exchange studied for at least three 1 hr. periods. In half of the experiments, two such series of three 1 hr. periods were obtained, separated by a few minutes in order to collect the urine by catheter.

Urines were analysed for sugar, nitrogen, creatine, and creatinine, as well as for acetone bodies, according to methods discussed previously [Barker *et al.* 1937]. The effects of exercise on urine constituents other

¹ The insulin was generously supplied by Eli Lilly & Co.

TABLE III. Details of a typical exercise experiment with a depancreatized dog

Period	Length of period hr.	B.Q.	Heat cal./hr.	Acetone mg./hr.	'D mg./hr.	N mg./hr.
Basal	1-00	0-721	27-50	—	—	—
	1-00	0-726	28-92	—	—	—
	1-00	0-731	28-78	—	—	—
	Average	0-730	28-40	266	1407	401
Exercise	0-90	0-762	64-27	265	1355	473
Recovery	0-75	0-668	32-01	—	—	—
	1-00	0-718	28-77	—	—	—
	1-00	0-708	28-56	—	—	—
	Average	0-720*	29-78	247	1604	394

* Weighted average B.Q. for exercise plus recovery.

than acetone have been discussed by Chambers *et al.* [1935] and by Lusk [1928], and our findings in these respects merely confirm their conclusions. Since the effects of D.N.P. have not been studied extensively on depancreatized animals, it may be stated that administration of this drug produces carbohydrate changes similar to those induced by epinephrine [Bollman, Mann & Wilhelmj, 1931; Chambers *et al.* 1935; Bachrach, Bradley & Ivy, 1936]. The glycosuria was nearly always increased for several hours, but the D : N ratios for later hourly periods, after the metabolism had returned to the normal rate, were much lower than the basal ratio, showing storage of carbohydrate in compensation for the previously increased glycosuria. There was a transient rise in the excretion of nitrogen, but no consistent changes in that of creatine or creatinine.

RESULTS

Exercise

The total increases in heat production and acetone excretion during the exercise and recovery periods from eighteen experiments on six dogs are shown in Table IV. The cal./hr. during the basal period were subtracted from the cal./hr. during exercise, and the difference multiplied by the length of time in hr. of the exercise in order to obtain the increase in heat production during the exercise period alone. By the same method, the increases in cal. and in ketone bodies were calculated for all experimental periods. The algebraic sum of exercise cal. and recovery cal. is given as the "Total increase in heat", with the corresponding changes in ketone for exercise plus recovery. As can be seen from the values tabulated, occasionally the heat production was lower during recovery than during the basal period. In these cases, the activity records

TABLE IV. Changes produced by oxalosis in metabolism of deuterioacetylated dogs

amount of acetylmethylated dogs

Dog no.	Urine			Excretion			Recovery			Total			Acetone yields				
	n.q.	Heat cal./hr.	Acetone mg./hr.	Increase in heat cal.	Increase in acetone mg.	Increase in heat cal.	Increase in acetone mg.	Increase in heat cal.	Increase in acetone mg.	n.q.	Increase in heat cal.	Increase in acetone mg.	Method 1		Method 2		
													Calc. mg.	Obtained %	Calc. mg.	Obtained %	
B 3	0.73	22.70	200	11.77	.47	-1.07	-80	0.72	10.10	-30	0.72	10.01	34	806	5	52	85
B 5	0.72	23.30	66	18.37	1.4	0.44	30	0.72	10.01	34	0.72	10.01	34	722	18	56	230
	0.73	23.53	78	13.81	0	2.22	132	0.73	17.03	132	0.73	17.03	132	078	4	10	120
	0.72	10.71	21	14.03	12	1.06	12	0.73	10.00	24	0.73	10.00	24	833	12	03	157
	0.72	17.75	57	15.51	10	4.14	80	0.72	10.05	90	0.72	10.05	90	023	35	212	154
	0.72	18.27	178	11.22	51	10.50	275	0.72	21.78	320	0.72	21.78	320	601	8	23	200
B 11	0.71	28.88	.47	22.87	0	-8.60	46	0.70	13.18	36	0.70	13.18	36	937	3	47	08
	0.70	21.73	36	17.35	-2	3.70	34	0.70	22.11	32	0.70	22.11	32	310	—	20	—
	0.71	23.35	91	11.16	-9	-3.41	-58	0.72	7.52	-07	0.72	7.52	-07	071	1	10	50
	0.71	16.34	7	18.34	-1	4.50	6	0.70	22.00	5	0.70	22.00	5	1083	1	16	34
	0.71	17.01	11	20.72	-2	3.82	9	0.71	25.51	7	0.71	25.51	7	273	87	24	088
B 13	0.70	31.38	118	0.01	8	-2.58	220	0.00	0.43	237	0.00	0.43	237	520	10	56	91
	0.70	27.70	125	18.38	5	-5.07	46	0.09	12.11	51	0.09	12.11	51	1100	—	256	—
B 14	0.73	28.40	266	23.32	0	4.04	-202	0.72	27.36	-202	0.72	27.36	-202	1252	—	333	—
	0.71	27.31	308	23.07	20	5.50	-170	0.70	20.53	-147	0.70	20.53	-147	1330	5	10	37
B 18	0.71	25.30	14	28.50	1	5.31	6	0.71	33.03	7	0.71	33.03	7	1772	1	71	35
	0.70	28.12	48	20.53	4	15.20	21	0.71	41.70	25	0.71	41.70	25	1130	3	78	50
0.71	28.30	82	28.22	0	-1.13	30	0.72	20.70	30	0.72	20.70	30					

indicated that the preliminary periods were not strictly basal in regard to movement.

The amount of increased ketosis to be expected was calculated in two different ways. (1) The first was from the increased amounts of fat oxidized, using the factor 0.36/8.5 g. of extra ketones for each additional cal. of heat liberated, as discussed earlier in this paper. Thus, the first exercise experiment on dog B 5 raised the heat production 19 cal. This would require the combustion of 2.6 g. of fat, which should yield 806 mg. of additional acetone bodies. Only 44 mg. extra, or 5 %, were obtained. In four instances the acetone excretion fell, even though the heat production increased considerably; furthermore, the acetonuria during later periods indicated in no case a compensatory rise. All the remaining experiments showed increases in ketone output, but, compared with the theoretical values, the amounts averaged about 10 % with one outstanding exception of 87 %. (2) The second method of calculation, from the basal proportions of acetone excretion and heat production, can be outlined as follows:

$$\frac{\text{Total increased cal.}}{\text{Basal cal.}} \times \text{basal acetone} = \text{expected increase in acetone.}$$

Since the R.Q.'s showed no change, it is probable that the same foodstuffs were being oxidized during the periods of increased metabolism as during the basal period [Canzanelli & Kozodoy, 1933; Chambers, Kennard, Pollack & Dann, 1932]. If each animal is deriving different amounts of ketone substances from fat, the ketosis should be increased in direct proportion to the rise in cal. This method of computation by direct comparison of exercise and basal periods would not be invalidated by the possibility that certain tissues (e.g. brain [Fazekas & Himwich, 1936], heart [Himwich, Goldfarb & Fazekas, 1936]) of the depancreatized animal may be oxidizing substances other than fat. When values calculated in this manner are used as standards, the amounts of extra acetone to be expected are considerably lowered; e.g. in the experiment mentioned earlier, 52 as compared to 806 mg. by the first method. The yields are thus higher, in this instance raising the amount recovered from 5 to 85 %, but, in general, are no more consistent. In five of the fourteen experiments which showed an increased ketosis, the values fall between 35 and 50 %; the remainder are spread out quite evenly up to 200 %, with one as high as 988 %. There are only two figures, 85 and 91 %, which fall into the range of "quantitative" recovery. It will be noted that more than one experiment was performed on five of the six dogs, and that the changes produced were not always quantitatively consistent in the same

animal. Macleod [1935, p. 716] stated that the ketosis of depancreatized dogs decreases uniformly after the first days of starvation. This cannot be the entire explanation of the present results, since animals B 5 and B 18 both excreted increasing amounts of acetone bodies as fasting continued, although B 11 and B 13 showed decreases. Similarly, certain of our long-fasted, depancreatized animals showed no consistent decline in ketosis, especially if there was no increased protein catabolism as the fasting was continued.

Since certain of the amino-acids comprising animal proteins are ketogenic in nature, it is of interest to mention that the nitrogen excretion increased in eleven of the fourteen cases in which the production of acetone rose over the basal level. Despite this qualitative trend, there does not seem to be any quantitative relationship between the catabolism of protein and the excretion of ketone bodies under the conditions of these experiments. For example, in the first experiment on dog B 5, with an increased acetone production of 44 mg., the additional nitrogen in the urine amounted to 33 mg. The next day the increased ketosis was 132 mg., but the urinary nitrogen during exercise and recovery fell 24 mg. below the basal value. Similar discrepancies in other cases lead one to conclude that the observed increases in ketosis did not arise from body protein.

Dinitrophenol

Table V summarizes the changes in metabolism occurring after injection of D.N.P. As in the exercise experiments, the level of R.Q. indicates that fat was the predominant foodstuff being oxidized both

TABLE V. Changes produced by dinitrophenol in metabolism of depancreatized dogs

Dog no.	Dinitrophenol							Acetone yields			
	Basal			Dosage mg./kg.	B.Q.	Increase in heat cal.	Increase in acetone mg.	Method 1		Method 2	
	R.Q.	Heat cal./hr.	Acetone mg./hr.					Calc. mg.	Obtained %	Calc. mg.	Obtained %
B 1	0.71	34.98	132	4	0.74	166.3	12	7051	1	626	2
B 3	0.73	21.75	96	4	0.72	52.3	-233	2218	—	231	—
B 6	0.71	28.39	315	4	0.73	134.8	-174	5716	—	1494	—
B 11	0.71	15.32	36	1	0.71	4.9	0	208	0	12	0
B 15	0.71	13.46	118	3	0.71	45.2	-62	1921	—	398	—
B 16	0.73	30.24	67	3	0.73	106.5	34	4516	1	236	14
B 16*	0.70	36.04	245	3	0.71	93.1	-311	3947	—	633	—
B 16	0.72	22.72	45	3	0.72	68.9	35	2921	1	137	26
B 17	0.74	18.31	23	4	0.71	82.1	-36	3481	—	103	—
B 17*	0.72	22.71	2	4	0.72	52.6	-8	2230	—	5	—
B 18†	0.72	37.33	29	4	0.72	301.0	-23	12762	—	234	—

* Animal in hyperthyroid condition.

† Animal in hyperthyroid condition, dinitro-ortho-cresol used.

during the periods of basal and increased metabolism. The increased outputs of heat and acetone were calculated as in the exercise series, and these are shown in the table, together with the theoretical yields of acetone calculated by the same two methods as before. Since the excretion of acetone during recovery was higher than during exercise in many instances, it was thought desirable to study two 4 hr. periods after injection of D.N.P. This was done in the last six experiments. The separate results are not shown in the table, however, since there were no marked differences in ketones. The increases for both periods were added, and the sum is shown in Table V under "Dinitrophenol".

Whichever standards are used, the amounts of additional ketone bodies excreted were less in every experiment than those calculated. Indeed, in only three of eleven instances were there any increases whatever in the excretion of acetone bodies, and in these the rise was so small as to be of little significance when compared with the tremendous increases obtained in oxygen consumption. The total increases in heat production caused by exercise average only about 20 cal., while those obtained following injection of D.N.P. lie between 50 and 170 cal., with one value of 300 after dinitro-ortho-cresol. This difference between the exercise and D.N.P. series, making especially prominent the lack of extra ketones in the latter, may be related to the fact that the drug heightened the animals' metabolism for several hours, in contrast to the exercise, the effects of which fell off rapidly after activity ceased.

Two of the D.N.P. experiments (as marked in the table) were performed on depancreatized dogs in the hyperthyroid condition. In one of these animals (B 16), superimposition of the drug upon hyperthyroidism produced a marked fall in ketosis, not encountered in either of the other two D.N.P. experiments with this dog. On the other hand, hyperthyroidism did not cause any significant differences in the response of dog B 17 to D.N.P. In the last experiment, on B 18, the largest increase in metabolism of the entire series was obtained, probably because dinitro-ortho-cresol was employed [Dodds & Pope, 1933].

Inasmuch as the present experiments were performed on intact, unanaesthetized, depancreatized dogs, for which data concerning metabolism of administered acetone bodies do not seem to be available, there are presented in Table VI the results of such experiments performed in this laboratory on three animals.¹ Since there were no significant changes in respiratory metabolism, these data are not included in the table.

¹ This portion of the work was supported through a grant from the American Association for the Advancement of Science.

TABLE VI. Recovery of acetone bodies after administration of approximately 5 g. (calc. as acetone) of the substance indicated

Dog no.	Acetone %	Acetoacetate %	β -OH butyrate %
B 20	94	84	75
B 24	34	49	74
B 35	44	29	30

About 5 g. (calculated as acetone) of each of the materials were dissolved in warm water and administered by stomach tube. Following this, urine for the estimation of ketone bodies, and expired air for acetone, were collected for about 12 hr. There is considerable variation among the different animals, the yields ranging from one-third to practically complete excretion of the compounds, representing from 1.5 to 5.0 g. acetone. The largest amount obtained in either the exercise or the D.N.P. series was 0.3 g., well below the amounts of administered ketone bodies excreted. It is probable, therefore, that the outputs of acetone bodies which were lower than the calculated values are not due to inability of the diabetic animal's kidney to excrete large amounts of these substances.

In the feeding experiments just mentioned, the amounts of acetone bodies not excreted, and presumably oxidized, were between 0 and 3.5 g. All of the amounts expected in the exercise series lie within this range, in contrast to six of the eleven D.N.P. experiments, in which the amounts of ketone bodies expected on the basis of the increased oxidation of fat were more than 3.5 g. However, no increased excretion of acetone substances was found in the latter series of experiments.

DISCUSSION

These results reveal that both exercise and dinitrophenol increase the oxidation of fat in depancreatized dogs without causing a correspondingly increased ketonuria. There are two obvious explanations for such a lack of correlation: first, that there is no stoichiometric production of acetone bodies from fatty acids, and, secondly, that the depancreatized dog is able to oxidize varying amounts of the four-carbon rests.

The first possibility is derived from much scattered evidence that forms of oxidation other than the well-substantiated progressive β oxidation may be involved in the catabolism of the fatty acids, thus leading to irregular production of the ketone bodies. Such suggestions have put forward α , γ , δ , ω , and "multiple alternate" oxidation as possibilities meriting consideration [cf. Sinclair, 1937]. In the present study one

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cannot determine how much each type of oxidation contributes to the final result, except to point out that in no case was there obtained more acetone bodies than would be expected on the basis of the formation of one four-carbon residue from each fatty acid. This suggests that such processes as "multiple alternate" oxidation do not take place to any great extent, since abnormally large amounts of the four-carbon rests would be produced by them.

The second possibility, namely, variabilities in oxidation of ketone bodies by the depancreatized animal may play fully as important a role. Beginning with Embden & Kalberlah [1906], many investigators have stated that the liver is the principal source of acetone bodies in the animal body. More recently, the belief that muscle is the site of oxidation of these substances has become important in explaining their ultimate fate. Chaikoff & Soskin [1928] found that resting muscle of eviscerated, depancreatized dogs could destroy injected acetoacetate as well as normal animals. Blixenkrone-Møller [1938] has also reported oxidation of ketone bodies derived from liver-perfusion by resting muscle of depancreatized as well as of normal cats. Friedemann [1936], from studies of acetoacetate perfusion, stated that, even after pancreatectomy, anaesthetized dogs were able to retain 95 % of considerable quantities of this substance infused over 4 hr. It is not clear, however, whether these animals were deprived of insulin over a sufficiently long period of time to render them completely diabetic.

Direct experiments under more physiological conditions were carried out by Himwich, Goldfarb & Weller [1931], who analysed conditions existing *in vivo* without the injection of ketogenic materials. Studying arteriovenous differences in anaesthetized, depancreatized dogs, they found that, although liver added large amounts of the four-carbon residues to the efferent blood, muscle also added ketone substances to the blood as often as it removed them. Furthermore [Goldfarb & Himwich, 1933], there was no correlation between the output from the liver of acetone bodies and their removal by striated muscle, cardiac muscle, or abdominal viscera. In a more recent study of arteriovenous differences of diabetic muscle from the same laboratory [Himwich, Goldfarb, Rakieten, Nahum & DuBois, 1934], it has been reported that a R.Q. below 0.7 was usually accompanied by a liberation of ketone bodies into the blood stream by the muscle, while an R.Q. above 0.7 occurred together with the removal of acetone. In this series of sixteen experiments, acetone bodies were liberated by muscle five times and removed eleven.

The above reports on resting muscle are supplemented by stimulation experiments. Blixenkron-Møller [1938], using a perfusate of livers from phlorhizinized, fasted cats as the source of acetone substances, found that the oxidation of these bodies was able to satisfy from 60 to 100 % of the energy requirements of hind-limb muscle preparations during electrical stimulation as well as at rest. Three depancreatized animals gave results in the same range; in these cases at least, the energy unaccounted for on the basis of ketone body oxidation (up to 40 %) doubtless came from the complete oxidation of fatty acids by the muscle. These results are in contradiction to those of Griesbach [1929], who claimed that electrically stimulated muscles of normal dogs utilized acetoacetate only at the same rate as resting muscle.

The administration of D.N.P. or of thyroid substance increases the rate at which large amounts of injected β -hydroxy butyric acid are destroyed by nephrectomized and by eviscerated rabbits, both acute preparations [Mirsky & Broh-Kahn, 1937]. The authors conclude that increased oxidation of ketone bodies by the extra-hepatic tissues would explain entirely the results of our experiments. It should be pointed out that the differences in species and in type of experimental preparation employed are probably important. The depancreatized animals used in the present studies were existing on the lowest plane of carbohydrate oxidation obtainable in a chronic preparation; consequently, any complication from this source was avoided.

In view of the evidence presented, the possibility still remains that two interacting factors determine ketone body excretion from fatty acids undergoing oxidation—namely, the breaking down of the carbon chains through processes which result in irregular production of the four-carbon rests, and the variable burning of these compounds themselves, once formed. From the evidence at present available, the evaluation of their relative importance is impossible.

In addition to the quantitative discrepancies discussed, the two series of experiments reported herein differ qualitatively from each other. Exercise, which doubtless increased the metabolism of muscle more than that of any other tissue, usually caused an increased output of acetone. In contrast, D.N.P. did not cause an increased ketosis, even though it stimulates skeletal muscle three times as much as it does liver [Alwall, 1936]. It has been contended that the liver not only produces the largest amounts of acetone bodies but also is unable to oxidize them [Snapper & Grünbaum, 1927; and others]. If this be accepted, then, in order to account for our results, it must be concluded either that D.N.P. causes

a specific alteration to take place in the course of oxidation of fatty acid by the liver, or that this drug enables the muscle to dispose of increased amounts of ketone bodies more efficiently than exercise. This qualitative difference reinforces the concept that excretion of ketone bodies is the resultant of production of these substances and their oxidation. The interrelation of these two factors can be completely changed by two different agents, exercise and dinitrophenol, both causing increased oxidation of fat.

SUMMARY

The depancreatized dog in the early stages of fasting was used in a study of the production of ketone bodies from fat, in order to eliminate antiketogenic factors.

In a large series of 3 hr. basal periods using seventeen depancreatized dogs, the actual excretion of acetone bodies amounted to about 10% of the amount calculated on the basis of one four-carbon residue from each fatty acid oxidized. In 80% of the experiments, the ketone excretion was less than that which could be derived theoretically from the protein being simultaneously broken down.

In eighteen exercise experiments the excretion of acetone bodies decreased in four, despite the increased oxidation of fat shown by studies of the respiratory exchange. In the rest, the increased excretion of ketone bodies could not be correlated with calculated yields.

In eight out of eleven experiments in which dinitrophenol was used to increase heat production, the excretion of ketone bodies either decreased or remained unchanged. The extra acetone obtained in the other three was but a small fraction of the calculated amount.

Possible explanations for these results are advanced, and it is considered that the findings may be due to a non-stoichiometric production of ketone substances from fatty acids in conjunction with variations in oxidation of these bodies once formed.

The author acknowledges with pleasure the invaluable aid in this work of Prof. W. H. Chambers and Prof. J. E. Sweet.

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CALCIUM AND SYNAPTIC TRANSMISSION IN A SYMPATHETIC GANGLION

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CALCIUM ions are necessary for the transmission of the excitatory state at the neuromuscular junction [Locke, 1894; Overton, 1904; Brown & Harvey, 1939] and at the ganglionic synapse [Bronk, Larrabee, Gaylor & Brink, 1938]. If acetylcholine (ACh.) is the chemical transmitter at these synaptic junctions, a failure of transmission there may be due either to deficient liberation of ACh., or to failure, as a result of altered threshold of the ganglion cell or motor end-plate, of the liberated ACh. to produce its normal effect. The perfused superior cervical ganglion of the cat is a convenient preparation for experiments designed to explore whether either of these possible defects, and if so which of them, is responsible for the failure of transmission which is produced by the withdrawal of calcium. Our experiments show that, when calcium is absent, there is no release of ACh. from the preganglionic nerve endings, either during stimulation of the sympathetic trunk or following the injection of potassium salts.

METHODS

Ganglion perfusion was carried out in the manner previously described [Feldberg & Vartiainen, 1934] with the aid of a small pump of the Dale-Schuster type, which provided a pulsatile pressure on the arterial side [MacIntosh, 1938]. The initial perfusion fluid was normal Locke's solution containing eserine sulphate in a concentration of 1:250,000. When it was desired to expose the ganglion to some modification of Locke's solution, the perfusion system was quickly rinsed and filled with the new solution: this could be done without changing the perfusion pressure or the temperature of the ingoing fluid. More transient effects were obtained by the injection of small volumes of solution into the perfusion fluid

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through the tip of the arterial cannula: solutions so injected were isotonic with Locke's solution. Small changes in osmotic pressure, of the order of those occasioned by the omission of calcium chloride from the Locke's solution, do not perceptibly modify the behaviour of the ganglion.

The movements of the nictitating membrane, which were registered isotonically with the eyeball removed, provided a continuous record of the activity of the ganglion cells.

The effluent fluid from the ganglion was assayed for ACh., either on the blood pressure of the cat in the manner described by Brown & Feldberg [1936], or on the dorsal longitudinal muscle of the leech, sensitized with eserine. In the latter case the ionic balance of the fluid was first restored, when necessary, by addition of isotonic solutions of the appropriate salts. Identical results were obtained with the two methods of assay.

RESULTS

In confirmation of previous work, the ganglion perfused with normal Locke's solution, containing eserine, discharged ACh. when effectively stimulated through the preganglionic nerve, and not at any other time.

The effects of low calcium

Spontaneous discharge of ganglion cells. When the perfusion fluid is changed for one containing no CaCl_2 , but with the other salts of Locke's solution and eserine in the usual concentrations, the cells of the ganglion

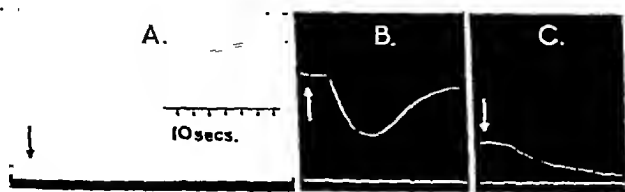


Fig. 1. Perfusion with Locke's solution containing eserine: contraction of nictitating membrane. A, at ↓, change to Ca-free Locke's solution. B, at ↑, injection of 0.2 c.c. normal Locke's solution. C, at ↓, change to normal Locke's solution. Between A and B 10 min., between B and C 20 min.

begin almost immediately to discharge (Fig. 1 A). The resulting contraction of the nictitating membrane may reach, within a few minutes, a level not far short of that attained with maximal stimulation of the preganglionic nerve at the rate of 10 per sec. The discharge may be maintained at nearly this level for as long as the perfusion with Ca-free solution is continued; or it may, in other experiments, gradually diminish,

and eventually almost disappear. Return to the original perfusion fluid immediately stops the discharge (Fig. 1C), which may also be temporarily inhibited by the injection into the arterial cannula of small volumes of Locke's solution (Fig. 1B) or of CaCl_2 solution: as little as 1 $\mu\text{g.}$ of Ca, injected in this way, can produce a perceptible relaxation of the contracted nictitating membrane.

A less intense discharge of ganglion cells occurs when the Ca content of the fluid is lowered, but not completely abolished. A very small contraction of the nictitating membrane follows the perfusion of a solution containing half the normal concentration of Ca, and larger contractions are recorded as the Ca content of the fluid is further lowered. The intensity of the discharge depends to some extent on the previous environment of the ganglion cells. Thus, in one experiment, perfusion of a solution containing 10% of the normal amount of Ca produced a contraction of the nictitating membrane which reached 50% of the height previously attained on maximal preganglionic stimulation; on changing to completely Ca-free solution, the contraction rose to 95% of maximal, then declined slowly to 50%; a second perfusion with the fluid containing 10% of the normal amount of Ca now reduced the contraction to 10% of maximal, whence it rose gradually to 30% of maximal, and remained at that level.

During the period of intense activity of ganglion cells, which accompanies perfusion of Ca-free Locke's solution, no trace of ACh. can be detected in the effluent fluid, although either of the tests used to identify it is usually sensitive to 0.001 $\mu\text{g.}$ of ACh., or about 2-3% of the amount liberated by 2 min. of maximal preganglionic stimulation when the perfusion fluid is normal Locke's solution.

Failure of synaptic transmission. Maximal stimulation of the preganglionic nerve, during perfusion with Ca-free Locke's solution, failed in two-thirds of our experiments to produce any peripheral effect (Fig. 2E). This failure of transmission occurred whether the ganglion cells, as indicated by contraction of the nictitating membrane, were already in intense activity, or had become less active, after prolonged perfusion with the Ca-free solution. In any case, the ganglion cells are not incapable of greater activity, for the injection of small amounts (e.g. 0.05 mg.) of KCl regularly produces a further contraction of the nictitating membrane. In three out of nine of our experiments preganglionic stimulation did, however, in the absence of Ca, result in a definite, though slight, increase in the response of the nictitating membrane. This effect, however, apart from its small dimensions, differed in several ways

from that mediated by the normal ganglion: the latent period was longer, up to 10 sec., and the contraction reached its maximum only after 1-2 min., subsiding also very slowly when stimulation was stopped.

No discharge of ACh. has ever been observed to accompany maximal preganglionic stimulation in the absence of calcium (Fig. 2E). This applies also to those experiments in which we observed the peculiar,

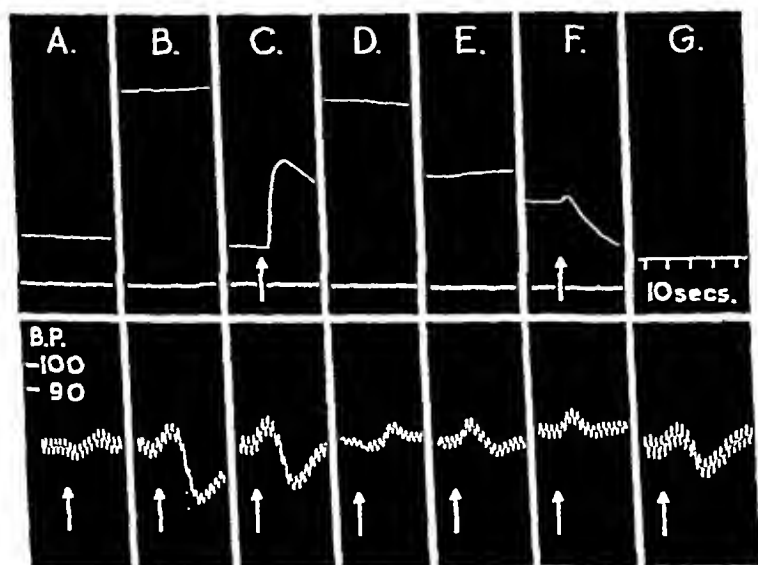


Fig. 2. Above, contraction of nictitating membrane; below, cat's blood pressure, effect of venous effluent collected during corresponding periods. A-C, perfusion with normal Locke's solution containing eserine: A, no stimulation; B, maximal preganglionic stimulation, 10 per sec.; C, at \uparrow , injection of 2 mg. KCl. D-F, perfusion with Ca-free Locke's solution containing eserine; D, no stimulation; E, 10 min. later, maximal preganglionic stimulation, 10 per sec., producing no further contraction of the nictitating membrane; F, at \uparrow , injection of 2 mg. of KCl. G, time signal, and effect of 0.005 μ g. of ACh.

small, delayed and slow peripheral response described above. It is possible that these occasional small responses may have been accompanied by release of ACh. in quantities too small for detection; alternatively, they may have been due to local persistence of calcium in the neighbourhood of occluded vessels, or to ionic changes at the synapse, increasing the frequency of the spontaneous ganglion-cell discharge.

Effect of injected potassium chloride. Absence of calcium increases the sensitivity of the ganglion cells to KCl injected into the perfusion stream

through the arterial cannula. In one experiment, for example, the dose of KCl just sufficient to produce a perceptible contraction of the nictitating membrane, when the perfusion fluid was normal Locke's solution, was 0.4 mg. When the perfusion fluid was changed to calcium-free Locke's solution, a larger response was produced by the injection of 0.1 mg. The ganglion cells are also sensitized to the paralysing effect of larger doses of KCl: thus, in the experiment just mentioned, the injection of 0.4 mg. of KCl, in the absence of Ca, produced a temporary relaxation of the contracted nictitating membrane.

We have never been able to detect any release of ACh. by injected KCl in the absence of Ca (Fig. 2F). The biological tests were sensitive enough to detect 20–25 % of the amount of ACh. liberated by the test dose of KCl with Ca present. The ability of KCl to discharge ACh. is therefore reduced by at least 75 %, and possibly further.

Effect of injected ACh. Perfusion with Ca-free Locke's solution raises the threshold of the ganglion cells for injected ACh. The cells apparently become less sensitive to the paralysing, as well as to the stimulating, effect of the drug.

The ACh. content of the ganglion. The fact that, in the absence of Ca, there is no spontaneous output of ACh., suggested that the failure of preganglionic impulses to release it was due to interference with the normal mechanism of release, and not to depletion of the depots. There was, however, a possibility that ACh. might have disappeared from the depots by some process unknown and undetected, and that the failure of preganglionic impulses was due to the resulting depletion. We tested this possibility by excising a ganglion which was being perfused with Ca-free Locke's solution, and comparing its ACh. content with that of the unperfused ganglion of the opposite side, the extracts being made with the aid of trichloroacetic acid in the usual way. The perfused ganglion was found to contain 0.50 μ g. of ACh. and the normal ganglion 0.42 μ g. The ACh. depots are therefore not depleted by Ca-free perfusion.

The decentralized ganglion. The cells of a ganglion, decentralized by section of the preganglionic trunk 1–3 weeks before the experiment, discharge impulses spontaneously during perfusion with Ca-free Locke's solution, just as do the cells of a normally innervated ganglion. Lowering of the threshold for injected KCl and raising of the threshold for injected ACh., and suppression of the spontaneous discharge by small amounts of Ca, can all be demonstrated in the decentralized as in the normal ganglion.

The effect of other changes in calcium and potassium concentration

The effect of perfusion with Locke's solution containing excess of K has been studied in detail by Brown & Feldberg [1936]. Such a solution produces a brief initial discharge of the ganglion cells, which then become inexcitable to indirect stimulation; ACh. continues to be discharged during this period of secondary paralysis. Prolonged perfusion with K-rich solutions eventually results in failure of the ACh. output also.

We have, in addition, varied the composition of the perfusion fluid in the following ways: (1) the Ca content was raised to twice or three times the normal; (2) K was omitted; (3) the fluid contained only 0.9% NaCl. Eserine was added in each case, in the usual proportion. The effects of these changes have not been investigated at length, since none of them caused any spontaneous discharge of ganglion cells, or prevented either the transmission of impulses through the ganglion, or the discharge of ACh. upon preganglionic stimulation. The few experiments we have made indicate that the excitatory and inhibitory effects of injected K-ions are reduced in the presence of excess of Ca-ions, and increased when K has been omitted from the perfusion fluid; the ability of injected KCl to discharge ACh. seems to be less affected by these changes. Prolonged perfusion with a solution containing five times the normal amount of Ca and the normal amount of K diminishes the effectiveness of preganglionic stimulation, without reducing the ACh. output. In one experiment we observed that, during perfusion with plain 0.9% NaCl, preganglionic stimulation for 2 min. was followed by a prolonged after-discharge of the ganglion cells.

DISCUSSION

Our experiments confirm the findings of Bronk, Larrabee, Gaylor & Brink [1938], that the absence of calcium ions causes a long continued spontaneous activity of the cells of a sympathetic ganglion, in the form of a repetitive discharge of impulses along the post-ganglionic axons, and, at the same time, a failure of the transmission of excitation from the synaptic endings of preganglionic fibres. Our experiments have shown that, when the absence of calcium has produced this condition, there is a corresponding failure of the output of ACh. normally evoked by preganglionic nerve impulses, or by potassium ions, so far as the available methods enable it to be detected. If the mobilization of potassium ions, accompanying a nerve impulse, can be regarded as the essential cause of the liberation of ACh. when the impulse reaches the preganglionic nerve endings, the two observations can be included in the single statement,

that, in the absence of calcium ions, potassium ions fail to produce their normal liberation of ACh. from the protective complex or precursor, in which it is held at the nerve endings.

Liberation of ACh. might be only one of a number of physiological processes in the ganglion requiring the presence of calcium ions, and it is necessary to enquire whether the withdrawal of calcium ions impairs the power of conduction in the intraganglionic portions of the preganglionic or the postganglionic fibres, or renders the ganglion cells inexcitable, before we can assess the significance of the failure of the acetylcholine release. With regard to the postganglionic axons and the ganglion cells, there is clear evidence from our own results that the former do not lose their power of conducting impulses and that the general excitability of the latter is certainly not impaired when calcium is withdrawn. The ganglion cells, as we have seen, exhibit a continued, spontaneous activity, discharging impulses which the postganglionic axons conduct. It might be suggested that this spontaneous activity, by producing the refractory state in a large proportion of the cells at any one moment, would interfere with their response to preganglionic stimuli; but this supposition is excluded by the fact that the responsiveness of the ganglion cells to sudden increase in the concentration of potassium ions, far from being diminished, is notably enhanced. It is, of course, not possible to test directly the conduction of impulses by preganglionic axons within the ganglion. There is evidence, however, that a lack of calcium ions renders nerve fibres in general abnormally excitable [Handovsky & Zacharias, 1924; Brink, Sjostrand & Bronk, 1939]; so that an assumption that the failure of transmission, when calcium is lacking, is due to defect of conduction or of response in nerve fibres or cells, would be not only unsupported, but against all the evidence which is available.¹ The only impairment of the sensitiveness of the ganglion cells by lack of calcium, for which we obtained any evidence, was an apparently specific, though not extensive lowering of their responsiveness to acetylcholine, observed when this was artificially applied. This, and the more complete and significant failure of the synaptic release of acetylcholine in detectable amount, are the only depressant effects of calcium deficiency on any ganglionic function, for which evidence has been found. It seems inevitable, therefore, to conclude that the failure of synaptic transmission

¹ Bronk [1939] has now reported experiments which show that removal of free Ca-ions from the stellate ganglion, by perfusion with citrate, produces spontaneous discharge in the preganglionic sympathetic fibres. It is clear, therefore, that the intraganglionic portions of these fibres, like the ganglion cells, are abnormally excitable when Ca-ions are absent.

is due to the failure of the transmitter, acetylcholine, to appear in active concentration. No other conception gives coherence to the facts observed. Brown & Feldberg [1935] showed that, under curarine, synaptic transmission failed because acetylcholine, though still released by pre-ganglionic impulses, no longer stimulated the ganglion cells; Kahlson & MacIntosh [1939] showed that, in the absence of glucose, synaptic transmission failed when the depot was depleted, so that there was no acetylcholine to be released; and the condition we have now described represents a third type of failure, in which acetylcholine cannot be released from the undepleted depots.

An absolute deficiency of calcium ions, in addition to a proportionate reduction in relation to potassium ions, seems to be necessary thus to prevent the liberation of acetylcholine, and also to produce the prolonged spontaneous activity of the ganglion cells. If a solution is perfused containing the proportion of calcium normal for Locke's solution, but a raised proportion of potassium, the result, as Brown & Feldberg [1936] observed, is a prolonged liberation of acetylcholine, and only a brief initial burst of impulses from the ganglion cells. On the other hand, perfusion with an isotonic solution containing only 0.9% sodium chloride, with neither potassium nor calcium ions, has practically no immediate effect on the function of the ganglion; there is no spontaneous activity of the ganglion cells, or liberation of acetylcholine; but when the pre-ganglionic nerve is stimulated the liberation of acetylcholine and transmission of excitation at the synapses occur normally.

In some recently published observations, Mann, Tennenbaum & Quastel [1939], working on brain slices *in vitro*, have found that the liberation of acetylcholine by potassium ions, comparable, one may suppose, to that which Brown & Feldberg [1936] had observed in the living ganglion, is reduced or abolished in the absence of calcium ions from the saline medium, as we have found it to be in the perfused ganglion. This is an addition to the points of correspondence already existing between the conditions governing the synthesis and liberation of acetylcholine in such preparations of surviving brain tissue, and those similarly operative in the perfused ganglion, where the function of acetylcholine as synaptic transmitter is supported by so much direct evidence.

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that, in the absence of calcium ions, potassium ions fail to produce their normal liberation of ACh. from the protective complex or precursor, in which it is held at the nerve endings.

Liberation of ACh. might be only one of a number of physiological processes in the ganglion requiring the presence of calcium ions, and it is necessary to enquire whether the withdrawal of calcium ions impairs the power of conduction in the intraganglionic portions of the preganglionic or the postganglionic fibres, or renders the ganglion cells inexcitable, before we can assess the significance of the failure of the acetylcholine release. With regard to the postganglionic axons and the ganglion cells, there is clear evidence from our own results that the former do not lose their power of conducting impulses and that the general excitability of the latter is certainly not impaired when calcium is withdrawn. The ganglion cells, as we have seen, exhibit a continued, spontaneous activity, discharging impulses which the postganglionic axons conduct. It might be suggested that this spontaneous activity, by producing the refractory state in a large proportion of the cells at any one moment, would interfere with their response to preganglionic stimuli; but this supposition is excluded by the fact that the responsiveness of the ganglion cells to sudden increase in the concentration of potassium ions, far from being diminished, is notably enhanced. It is, of course, not possible to test directly the conduction of impulses by preganglionic axons within the ganglion. There is evidence, however, that a lack of calcium ions renders nerve fibres in general abnormally excitable [Handovsky & Zacharias, 1924; Brink, Sjostrand & Bronk, 1939]; so that an assumption that the failure of transmission, when calcium is lacking, is due to defect of conduction or of response in nerve fibres or cells, would be not only unsupported, but against all the evidence which is available.¹ The only impairment of the sensitiveness of the ganglion cells by lack of calcium, for which we obtained any evidence, was an apparently specific, though not extensive lowering of their responsiveness to acetylcholine, observed when this was artificially applied. This, and the more complete and significant failure of the synaptic release of acetylcholine in detectable amount, are the only depressant effects of calcium deficiency on any ganglionic function, for which evidence has been found. It seems inevitable, therefore, to conclude that the failure of synaptic transmission

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ON THE DISAPPEARANCE FROM THE BLOOD OF INTRAVENOUSLY INJECTED INSULIN

BY H. K. GOADBY¹ AND J. S. RICHARDSON

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(Received 1 September 1939)

It is not yet universally agreed which of the two hypotheses about the secretion of insulin by the pancreas is correct; whether insulin is periodically secreted in response to a rise in the blood-sugar concentration; or whether there is a continuous secretion at a constant rate, a more recent view put forward by Soskin, Allweiss & Cohn [1934].

In either case, a certain amount of insulin is required daily by the body. Furthermore, when normal men or animals, or a diabetic patient, or an animal with experimentally produced diabetes, are injected with insulin, this insulin appears to work only for a limited time. It seems then that, whichever hypothesis as to the secretion of insulin is right, there is apparently a mechanism whereby insulin is either inactivated or destroyed, or removed from the body. The experiments to be described in this paper were designed to enquire into such a mechanism.

Briefly, the apparent disappearance from the circulating blood of intravenously injected insulin was observed in normal animals, in animals with the liver excluded from the circulation, or with the kidneys so excluded, or after the injection of Young's glycotropic hormone of the pituitary.

METHODS

Rabbits of 1.5-2.8 kg. were used throughout. The experiments to observe the disappearance of insulin from the circulation of normal, intact rabbits and of operated animals, were performed in exactly the same way; the technique was as follows:

1. The rabbit *A*, to be tested for insulin disappearance, was first given 10 g. glucose in 20 c.c. water by stomach tube in order to prevent hypoglycaemic reactions occurring as the result of the large dose of

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2. The omission of calcium from the perfusion fluid, other cations being present in normal concentrations, has the following effects:

(a) The ganglion cells discharge spontaneously.

(b) Synaptic transmission fails, owing to the failure of preganglionic impulses to liberate acetylcholine.

(c) The ganglion cells are sensitized to both the stimulating and the paralysing actions of injected potassium chloride.

(d) The response of the ganglion cells to injected acetylcholine is diminished.

3. These results are not obtained on perfusion with isotonic NaCl solution, or with solutions containing excess of potassium, but calcium in normal concentration.

We wish to thank Sir Henry Dale for his stimulating interest in this work.

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around the pedicle to include the artery and vein, but not the ureter, and is tied in a loose half hitch. With needles the threads are then passed, one through the posterior abdominal wall to come out through the skin about 2 in. from the midline as nearly as possible dorsal to the kidney, the other through the anterior abdominal wall, so that it lies directly ventral to the kidney after the incision is closed. The intestines having been retracted to the other side, a ligature is similarly passed round the other renal pedicle, and the two thread ends passed out through the abdominal wall. The abdomen is then closed in two layers, the peritoneum plus muscle and the skin, by continuous sutures. Each pair, right and left, of threads is tied together so as to lie easily round the side of the animal. The wound is covered with a dressing of gauze soaked in collodion. The threads are protected from chewing or rubbing by covering them with a piece of adhesive strapping right round the abdomen. In a warm cage the animals soon recover. Four days later, the insulin experiment is performed. The strapping is carefully removed, exposing the threads; each pair, dorsal and ventral, is untied or cut, and then pulled firmly and steadily until quite tight. The ends are then cut off. The animals seem quite undisturbed by this procedure.

That the ligatures are tight around the renal pedicles and remain so, thus excluding the kidneys from the circulation, is proved by (i) the rabbits pass no urine until death; (ii) at post-mortem, the lamp-wick ligatures are tight on the renal pedicles and the kidney parenchyma is degenerated.

Method of expressing results. The criterion had first to be established of what is the minimum drop in blood-sugar concentration in rabbits that can be taken as evidence that the injected blood has a hypoglycaemic action, and therefore presumably contains insulin. (For the sake of brevity in this paper this action of the blood samples in depressing the blood sugar will be called the insulin action.)

The blood sugars of ten normal rabbits (18-20 hr. fasting) were estimated at 10 min. intervals for 80 min., the period of the standard experiment. These experiments, Fig. 1, showed that owing to considerable spontaneous variations in the blood-sugar concentrations obtained under these conditions and by this technique, insulin action of injected blood can only be inferred if the blood-sugar concentration falls in a smooth curve to more than 20 mg. below the lowest of the three pre-injection values. Therefore, a blood will be said to have given a "positive reaction" only if this occurred; irregular drops or variations of less than 20 mg. are called negative.

insulin to be given; 5-20 min. later it received an intravenous injection of 40 units of insulin (1 c.c. of Boot's "double strength" ordinary insulin). From time to time after this, successive 5 c.c. samples of blood were taken from an ear vein directly into three drops of 20% potassium oxalate solution in a small tube. The blood was mixed at once to prevent clotting and kept at room temperature. (Preliminary experiments showed that the hypoglycaemic action of the blood with insulin added *in vitro*, or of blood taken whilst insulin is circulating *in vivo*, is not diminished on keeping at room temperature or 37° for at least 6 hr., the maximum time it was kept before testing in any experiment.)

II. Three other rabbits *B*, *C* and *D*, which had been starved 18-24 hr., were placed in their boxes and allowed 5-20 min. in which to settle down. From each, three preliminary blood samples for sugar content were then taken at 10 min. intervals. After this, 5 c.c. of the first sample of oxalated blood from rabbit *A* were injected into the marginal ear vein of *B*; blood was then taken for blood-sugar estimation on *B* at 10 min. intervals for 50 min. The same process was repeated on rabbit *C*, with the second sample from *A*, and on *D* with the third sample.

Thus the depression of the blood-sugars of rabbits *B*, *C* and *D* would indicate the presence of insulin in the blood samples from rabbit *A*, and therefore its presence in the circulation of *A* at stated times after it was injected intravenously.

Blood sugars. These were estimated on single 0.1 c.c. samples of blood taken from a marginal ear vein by the method of Hagedorn & Jensen [1923].

Exclusion of the liver from the circulation. This was done by following exactly the technique of Himsworth [1938], 3 weeks being left between the first and second operations, 4 days between the second operation and the insulin experiment.

Pituitary glycotropic hormone. This was kindly made by Dr F. G. Young, to whom the author is greatly indebted. Four c.c. of the sample provided were injected 18 hr., and 4 c.c. 2 hr. before the experiment, thus following exactly his recommended procedure [1936].

Exclusion of the kidneys from the circulation. A technique was evolved similar to the one used for exclusion of the liver. Under ether anaesthesia, with full aseptic precautions, the rabbit's abdomen is opened, the intestines are retracted to one side, and the kidney exposed to view. A ligature is ready, consisting of 2 in. of soft, round lamp-wick, to each end of which about 6 in. lengths of No. 18 thread are tied. By blunt dissection, the renal pedicle is exposed in the peritoneal fat, the ligature is slipped

that if a negative reaction is obtained from a blood sample it contains probably less than $\frac{1}{30}$ unit in the volume (5 c.c.) injected.

A positive reaction indicates that more than $\frac{1}{30}$ unit is present. From the blood-sugar depression curves obtained, it was found impossible by a single experiment on a rabbit to obtain anything approaching an accurate measure of the dose given, whatever the method of calculation used. This is, of course, to be expected in animal experiments, and is in agreement with the findings of all workers on the biological assay of insulin.

Disappearance of insulin action from circulating blood

In intact rabbits (twenty-one animals). Seventeen of these had blood taken immediately, i.e. 1-5 min. after insulin injection, eleven 30 min., eleven 60 min., and seven 90 min. later. Table II and curves 2, 3, 4 and 5, Fig. 2, show that in normals, blood taken immediately after the

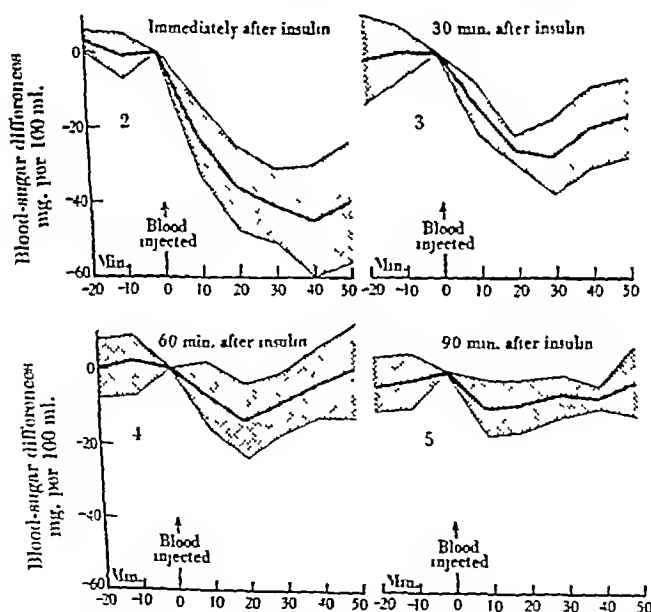


Fig. 2. Disappearance of insulin from blood in normal animals. Curves of blood-sugar depression following transfusion. Donors=normal rabbits. Bloods taken 2, 30, 60 and 90 min. after 40 units insulin intravenously.

injection of 40 units of insulin intravenously, has a strong insulin action. In the 30, 60 and 90 min. samples, this action gradually diminishes; at 90 min. all seven tests were negative.

The results are also expressed in the form of curves; in these, the third pre-injection blood sugars are taken as 0; all the other values in any one experiment are calculated as mg. plus or minus this reading. The points on the curves shown are the means of all experiments in a group; the shaded areas are drawn to include plus and minus the probable

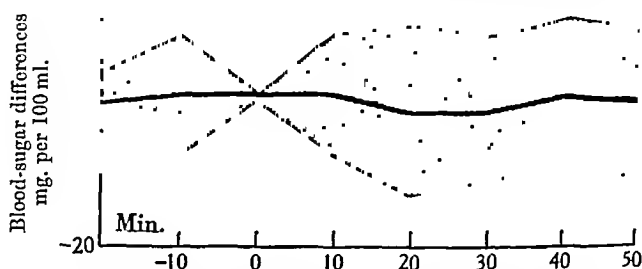


Fig. 1. Blood-sugar variations in normal, fasting rabbits.

error of each man. This "probable error" is obtained from the formula $\frac{3 \times \sigma}{\sqrt{n}}$, where σ is the standard deviation of the blood-sugar difference values, n the number of experiments; the factor 3 is taken in place of the more usual 2 because of the small size of the samples.

RESULTS

Preliminary. The first problem was to find out what is the minimum dose of actual insulin which gives a "positive reaction" in normal rabbits; the experiments are summarized in Table I. From these figures

TABLE I

Insulin units	Blood-sugar depressions	
	Positive reactions	Negative reactions
1.0-0.5	4	—
0.33-0.1	5	1 (16 mg. fall)
0.075-0.029	7	1 (13 ")
0.027	—	1 (19 ")
0.025	2	—
0.020	1	4
0.015	1	1
0.0125	1	3
0.010	—	1

Sensitivity of normal fasting rabbits to intravenous insulin. Blood-sugar reactions to diminishing doses.

it may be concluded that the limit of sensitivity of the rabbits under the conditions and criteria of the experiments is about 0.03 unit. This means

the three blood samples be obtained from the ear vein. In three more, the third blood sample (60 min.) was taken direct from the heart after

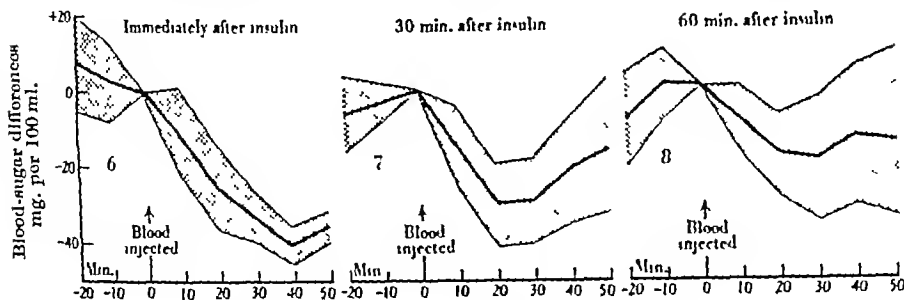


Fig. 3. Blood-sugar depression. Donors = liver-excluded group.

opening the chest, the rabbit being moribund. Four animals did not survive much longer than 30 min., and in two of these, heart blood was then taken.

This failure of the animals to survive very long after exclusion of the liver was disappointing in comparison with Hims-worth's experiments, in which over 4 hr. was the average survival time. The rabbits in the author's experiments appeared to die of shock; only in one case was any other obvious cause found post-mortem, namely haemorrhage from a torn hepatic vein. Five out of eleven animals in which the liver was excluded died in convulsions: these were not due to hypoglycaemia, their blood sugars during convulsions were 119, 162, 138, 104, 91 mg./100 c.c. respectively. In five rabbits which died of shock and did not have convulsions, the blood sugars were 161, 49, 167, 88, 32.

Table II and curves 6, 7 and 8, Fig. 3, show that the disappearance of insulin action proceeds at the same rate when the liver is excluded from the circulation as it does in the normal, intact animals: the differences in the figures are too small to be statistically significant. On five rabbits the insulin experiment was

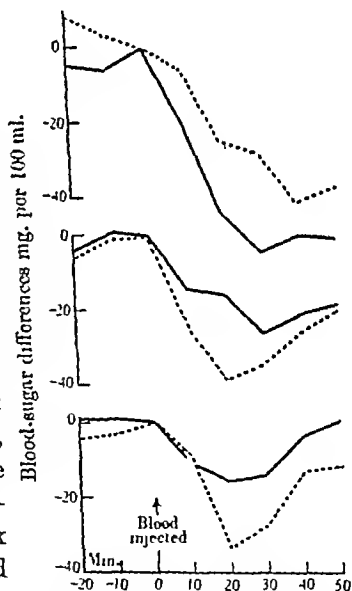


Fig. 4. Mean curves from five rabbits before and after exclusion of the liver. Top curve = results of bloods taken immediately after insulin. Middle curve = 30 min. after insulin. Bottom curve = 60 min. after insulin. Continuous line = before liver exclusion. Dotted line = after liver exclusion.

TABLE II

Groups	Times after insulin injection of taking blood samples			
	2-5 min.	30 min.	60 min.	90 min.
Normals	100 (17)	82 (11)	40 (10)	0 (7)
After first operation on liver	100 (6)	80 (5)	17 (6)	—
Livers excluded	100 (11)	73 (11)	57 (7)	—
Kidneys excluded	100 (10)	—	90 (10)	70 (10)
Glycotropic hormone	100 (4)	50 (4)	25 (4)	—

Insulin action of bloods taken at various times after injection of insulin in the various experimental groups. The main figures denote the percentage of "positive reactions" obtained, the figures in parentheses are the numbers of experiments in the groups.

That this insulin action is due to the exogenous insulin, and not to endogenous insulin secreted in response to the glucose given, was controlled by experiments in which normal rabbits were simply given glucose by stomach tube, and their blood tested for insulin action afterwards. In one animal, blood was taken 2 min. after the glucose; this gave a just positive reaction; the figures of the blood-sugar depression of the rabbit on which this blood was tested were -7, -14, -24, -19, -10 mg. at successive 10 min. intervals after receiving the blood. By comparison with curve 2, Fig. 2, of the results immediately after injection of insulin, it will be seen that the points lie well outside the probable error of the mean depression curve. Two animals had blood taken 23 min. after glucose only; both gave negative results, as compared with nine positive out of eleven 30 min. after insulin. Two animals gave completely negative results 50 min. after glucose only. No further control experiments were done, as it was thought that the insulin concentration in the blood in response to glucose would be outside the limit of sensitivity of the method of testing for it. This supposition was strengthened by the facts that the one positive response to glucose only was so small, and that it was known that up to 1 hr. after giving the glucose, the blood sugars were at high enough levels to be adequate stimuli for the secretion of endogenous insulin; and yet after 23 and 50 min. all tests were negative. It can, therefore, be reasonably assumed that the insulin action of blood taken after intravenous injection of insulin, as tested by the present technique, is due to the exogenous insulin.

After exclusion of the liver (11 rabbits). The glucose was given first; after 5-10 min. the ligatures were pulled tight, and after 1-3 min. more, the insulin injected. When possible, blood was taken 2, 30 and 60 min. after the insulin. The tightening of the ligatures and the injection of insulin were followed by severe shock, so that in four cases only could

positive insulin action 90 min. after injection of insulin. This is a statistically significant difference, as is also the number of positive reactions after 60 min. Table II and curves 12, 13 and 14, Fig. 6, illustrate this. The rabbits survived 1-4 days after the experiment; at the time of the insulin experiment they appeared quite normal and undisturbed.

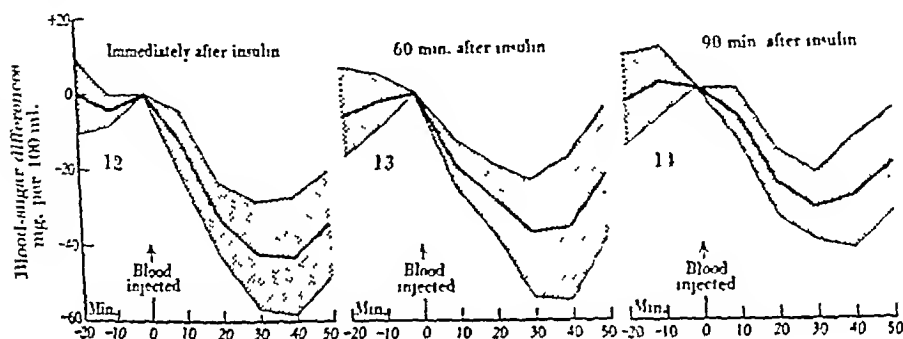


Fig. 6. Blood-sugar depression. Donors = kidney-excluded group.

Blood-sugar concentrations of the rabbits after glucose and insulin. The mean blood sugars of the various groups of insulin-injected rabbits are given in Table III. From these figures it can be seen that the glucose effectively prevents any hypoglycaemia during the period of the experiments, even in the animals with the livers tied off; in these the glucose can only be absorbed into the general blood stream via the collateral circulation established by the first operation.

TABLE III

	Times after insulin injection		
	2 min.	30 min.	60 min.
<i>Normals</i>	204 (30)	190 (78)	185 (76)
<i>Livers excluded</i>	131 (30)	134 (61)	122 (68)
<i>Kidneys excluded</i>	208 (26)	134 (39)	155 (41)
<i>Glycotropic hormone injected</i>	231 (39)	286 (57)	280 (56)

Blood sugars of rabbits after glucose by stomach tube and insulin intravenously; concentration in mg./100 c.c.; figures in parentheses = standard deviations.

The blood sugars of the liver-excluded group lie at a consistently lower level than in the normals, whereas those of animals receiving glycotropic hormone are at a higher level. This may be a true finding and is *a priori* to be expected. However, on the criterion that for a difference between the means to be significant it must be greater than three times the standard error, the differences obtained can all be due to errors of sampling. The only exceptions are: (1) The liver-excluded rabbits

done both between the first and second operations, i.e. with the portal vein partly obstructed, and also when the liver was completely excluded from the blood stream. The results are given in Fig. 4. It may be thought that these curves indicate that the disappearance of insulin action is somewhat slower after exclusion of the liver: but the condition of shock of the animals, especially in view of the results after exclusion of the kidneys to be described later, makes it impossible to lay any stress on the somewhat small differences obtained. To sum up, there is no evidence from these experiments that exclusion of the liver from the circulation has any effect on the rate of disappearance of the hypoglycaemic action of the circulating blood after intravenous injection of insulin.

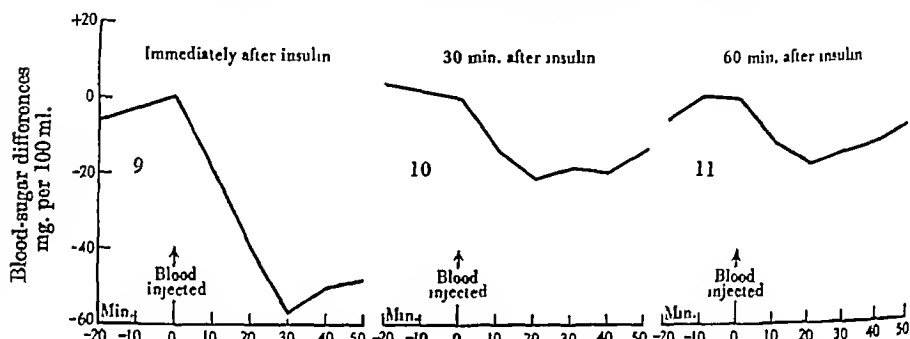


Fig. 5. Blood-sugar depression. Donors = glycotropic-hormone injected group (mean values only).

Glycotropic hormone. The doses of the hormone given were assumed to be such as to render the rabbits insensitive to the hypoglycaemic action of at least 3 units of insulin intravenously. Evidence that the hormone was active in this respect was afforded by the fact that in all four rabbits the blood sugar rose markedly 30 min. after the glucose and insulin were given; this happened only in four out of ten normals, three out of nine with livers excluded, in none out of six with kidneys excluded: the action of insulin appeared thus to be inhibited to some extent. Only sufficient hormone for four animals was available; the results do not differ from those of normals, as seen in curves 9, 10 and 11, Fig. 5. In these only the means of the four experiments are plotted.

After the exclusion of the kidneys (11 experiments). This group shows that the insulin action persists longer in the blood stream of animals in which the kidneys are excluded from the circulation, than in that of normal, intact animals. Of the animals so treated 70% showed a

positive insulin action 90 min. after injection of insulin. This is a statistically significant difference, as is also the number of positive reactions after 60 min. Table II and curves 12, 13 and 14, Fig. 6, illustrate this. The rabbits survived 1-4 days after the experiment; at the time of the insulin experiment they appeared quite normal and undisturbed.

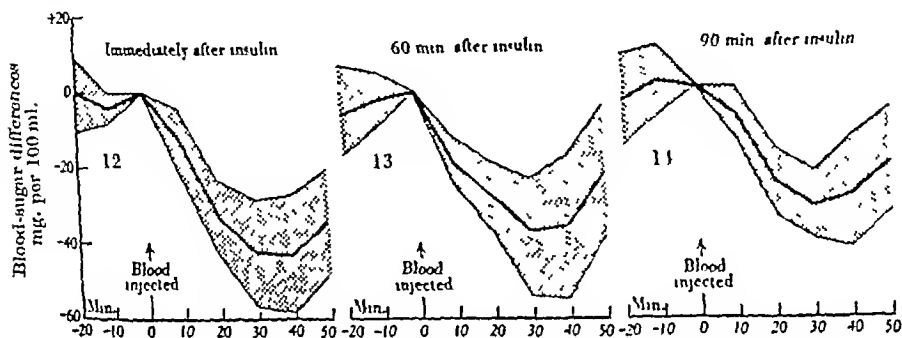


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immediately after injection of insulin had lower blood sugars than all the other groups at the same time; this presumably indicates slower absorption of glucose into the general circulation, as the blood is taken after glucose has been given, before the insulin has had time to act. (2) The liver-excluded and the glycotropic-hormone injected groups show a considerable difference throughout; owing to the very small number in the latter group, no emphasis will be laid on this difference.

DISCUSSION

Blood taken from a rabbit immediately after intravenous injection of 40 units of insulin depresses the blood sugar of normal fasted rabbits. This is in agreement with the findings of Heymans & Heymans [1927]; they found that blood taken within 4 min. of intravenous injections of 2-15 units of insulin occasionally produced hypoglycaemic reactions in other rabbits. Kepinow & Ladept-Petit Dutailis [1927a], however, failed to demonstrate on dogs, any blood-sugar depressing action of 360 c.c. of blood from other dogs, taken at various times down to $\frac{1}{2}$ min. after the intravenous injection of 12 units of insulin; these doses of insulin are much smaller than those used by the authors.

This insulin action, present immediately after injection of insulin, gradually diminishes, and by 90 min. has almost, if not quite vanished. Now a 2 kg. rabbit has about 200 c.c. of blood; also it has been shown above that the insulin action of the circulating blood in these experiments is due only to the injected insulin. Its concentration at the start can theoretically be 40 units in 200 c.c. (1 unit in 5 c.c.); in 90 min. this has fallen to less than $\frac{4}{5}$ units in 200 c.c. ($\frac{1}{5}$ unit in 5 c.c.).

The disappearance of the action is unaffected by exclusion of the liver from the circulation, a finding in contrast with that of Kepinow & Ladept-Petit Dutailis [1927b]. These workers anastomosed the portal vein to the renal vein of a dog; with the liver thus removed from the circulation, they demonstrated insulin in the circulating blood, 3-20 min. after 12 units of insulin given intravenously. By comparison with their first experiment they concluded that the liver inactivates the insulin or removes it. However, the authors' experiments indicate that the liver is not necessary for the gradual disappearance of the insulin action of the circulating blood after a large dose of insulin is injected intravenously; glycotropic hormone seems also to have no effect on this disappearance. Exclusion of the kidneys, however, from the circulation results in a longer persistence of the insulin action in the blood; this suggests that the kidneys are responsible for the removal of insulin from the blood when it

is present there in high concentration. Fisher & Noble [1923] in the early days of insulin asserted that they could recover from the urine 35 out of 40 units of subcutaneously injected insulin. Partos [1932], by an extraction method, obtained insulin from the urine of all subjects tested except human diabetics, pancreatctomized cats, and rabbits fasted for more than 48 hr. Burger & Friedman [1938] injected rabbits' urines intravenously into other rabbits. They found that there was no insulin action in normal rabbit urine; it was present there up to 2 hr. after the intravenous injection of 200 units of insulin. Recordier & Andriac [1935] found that bilateral nephrectomy resulted in the persistence up to 100 min. of the action of 2 units of insulin injected intravenously, whereas in the same rabbits before nephrectomy, its action was over by then.

The amount of insulin circulating in the blood of a rabbit after 40 units of insulin are injected intravenously is probably far in excess of normal physiological limits; therefore the mechanism for inactivating or getting rid of this may not be the one which deals with insulin normally secreted by the pancreas. It is hoped with the aid of a more sensitive test animal to extend these observations to concentrations of insulin more nearly physiological, and safely attainable by injection of insulin in human beings.

SUMMARY

1. After the injection of 40 units of insulin intravenously into rabbits, it is possible to show that the circulating blood has a blood-sugar depressing action, by transfusing it into other fasted rabbits; this action is shown to be due to the injected insulin.
2. This hypoglycaemic action gradually disappears and has gone by the end of about 90 min.
3. The rate of disappearance is not affected by excluding the liver from the circulation, nor by the previous injection of glycotropic hormone of the pituitary gland.
4. Excluding the kidneys from the circulation results in a persistence of the insulin action.
5. The liver, therefore, is not responsible for the inactivation or removal of large doses of exogenous insulin from the blood stream; the kidneys are concerned in such a mechanism.

immediately after injection of insulin had lower blood sugars than all the other groups at the same time; this presumably indicates slower absorption of glucose into the general circulation, as the blood is taken after glucose has been given, before the insulin has had time to act. (2) The liver-excluded and the glycotropic-hormone injected groups show a considerable difference throughout; owing to the very small number in the latter group, no emphasis will be laid on this difference.

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A CIRCULATION MODEL

BY L. E. BAYLISS

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THE model to be described is an ideal separation of the components of the mammalian circulation which are responsible for the effects of "capacity" and of "resistance". The most essential point about it is that it has a closed circuit and is such that the rate of flow of fluid round it depends upon the degree of filling.

The pump (Fig. 1) consists of a flaccid rubber tube clamped at one end and fitted to a T-piece at the other, rhythmically compressed by any suitable means. (About 5 in. of bicycle inner tube serves very well.)

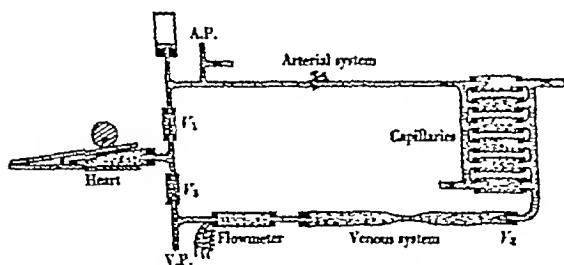


Fig. 1. Diagram of circulation model. V_1 , V_2 , V_3 , valves; A.P., V.P., connexions to manometers for arterial and venous pressures respectively.

The degree of filling during "diastole", and hence the amount ejected during the subsequent "systole", depends on the "venous" pressure and the resistance to flow of the input valve. A sufficiently compliant "venous" system can be obtained by using two cylindrical toy balloons with their ends cut off joined together in series. The "capillary" system, with large and variable volume, but negligible resistance to flow, consists of two six-way manifolds, made from $\frac{1}{8}$ in. brass gas T-pieces, connected together by six thin-walled rubber tubes of about 1 in. diameter and 4 in. long. (These can be made out of sheet rubber—there

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It should be noted that, in all these procedures, the venous pressure and circulation rate always move together; the arterial pressure is independently variable and is no necessary indication of the circulation rate.

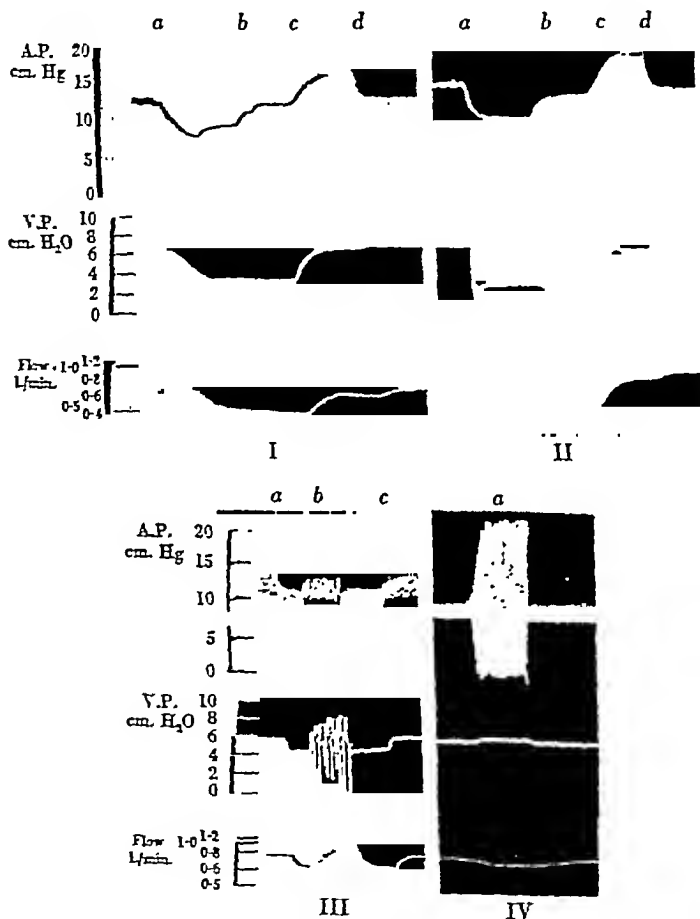


Fig. 2. Records obtained with circulation model. From above downwards: arterial pressure in cm. Hg (membrane manometer); venous pressure in cm. H₂O (membrane manometer); rate of flow in litres per min. (the absolute values are somewhat uncertain owing to zero drift in the recorder). I: (a) haemorrhage; (b) arteriolar constriction; (c) intravenous infusion; (d) arteriolar dilatation. II: (a) capillary dilatation; (b) arteriolar constriction; (c) intravenous infusion; (d) arteriolar dilatation. III: (a) arteriolar constriction; (b) veins massaged; (c) capillaries raised to level of heart. IV: (a) elastic cushion clamped off.

IV. At (a) the elastic cushion is clamped off; this leads to an enormous increase in the pulse pressure, the arterial pressure falling to zero on each

is no appreciable pressure within them, so that the seams need not be very carefully made.) They are ordinarily more or less flattened out by being compressed by two cylindrical lead weights, each about 1 in. diameter and 3 in. long. "Capillary dilatation" is brought about by removing one or both of these weights. The "arterial" system consists of (a) an inverted bottle as used for the "elastic cushion" in the apparatus associated with the heart-lung preparation, followed by (b) a rubber tube with a screw clip ("arterioles"). Side tubes are provided for registering the arterial and venous pressures; the other side tubes shown in Fig. 1 are useful for filling the model and for "bleeding" and "intravenous injection". It is highly desirable to include some form of flowmeter in the circuit, so that changes in the rate of flow can be directly recorded. The records given in Fig. 2 were obtained by means of a thermo-electric flowmeter similar to that described by Winton [1936].

In Fig. 2 are given records obtained on the model showing the responses to the following procedures:

I. (a) Haemorrhage, followed by (b) arteriolar constriction; the arterial pressure is restored, but not the circulation rate, so that the tissues may still be inadequately supplied with oxygen. At (c) an intravenous infusion is given, and at (d) the arterioles are dilated, so that the original conditions are restored. It will be noticed that constriction of the arterioles results in a small fall in venous pressure and circulation rate, and that dilatation results in a small rise; this is due to the change in the amount of fluid in the elastic cushion which follows a change in the arterial pressure, and which must be associated with an inverse change in the amount of fluid in the venous system. This effect must occur to some extent in the normal animal, but is very much smaller, owing to the greater compliance of the venous system.

II. (a) The capillaries are made to dilate, and at (b) the arterioles are made to constrict so as to restore the arterial pressure; again, the circulation rate is not restored, but slightly reduced. At (c) an intravenous infusion is given, which restores the venous pressure and circulation rate to the initial values. At (d) the arterioles are made to dilate and the initial conditions are completely restored, even though the capillaries remain dilated.

III. (a) The effect of gravity is shown by lowering the capillary end of the model a few inches below the heart end. There is a fall in venous pressure, circulation rate and arterial pressure. (b) The veins are massaged rhythmically with restoration of both pressures and the circulation rate. At (c) the capillary end of the model is restored to its original position.

It should be noted that, in all these procedures, the venous pressure and circulation rate always move together; the arterial pressure is independently variable and is no necessary indication of the circulation rate.

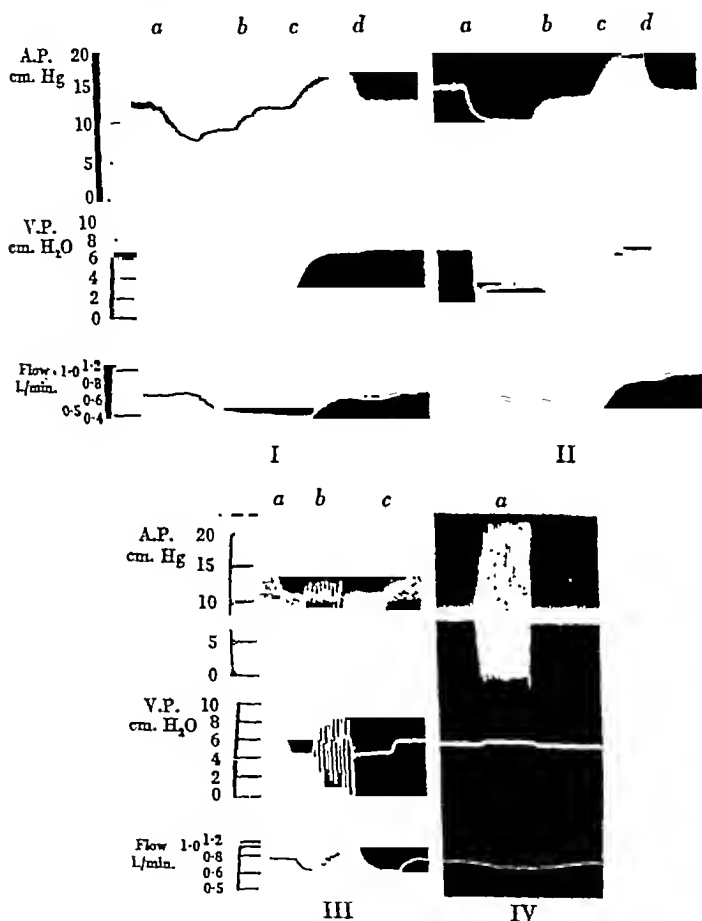


Fig. 2. Records obtained with circulation model. From above downwards: arterial pressure in cm. Hg (membrane manometer); venous pressure in cm. H₂O (membrane manometer); rate of flow in litres per min. (the absolute values are somewhat uncertain owing to zero drift in the recorder). I: (a) haemorrhage; (b) arteriolar constriction; (c) intravenous infusion; (d) arteriolar dilatation. II: (a) capillary dilatation; (b) arteriolar constriction; (c) intravenous infusion; (d) arteriolar dilatation. III: (a) capillaries lowered below level of heart; (b) veins massaged; (c) capillaries raised to level of heart. IV: (a) elastic cushion clamped off.

IV. At (a) the elastic cushion is clamped off; this leads to an enormous increase in the pulse pressure, the arterial pressure falling to zero on each

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THE FORMATION OF URINE IN THE AMPHIBIAN
AND MAMMALIAN KIDNEY

BY P. ELLINGER

*From the Lister Institute, London**(Received 31 December 1937)*

THE present communication is concerned with the function of the amphibian and the mammalian kidney as revealed by intravital microscopic examination of its responses to certain dyes and drugs. The aim of the experiments has been to discover whether the tubules have a secretory function in addition to their reabsorptive activity, and if so, whether this is a normal function of the proximal convoluted tubules or one which comes into play only under unusual conditions.

The mechanism of glomerular filtration and tubular reabsorption, postulated by the theory of Ludwig and Cushny, has been largely recognized [cf. Smith, 1937]. It has been confirmed in recent research on the function of the vertebrate kidney, especially by a study of the chemical composition and the physico-chemical properties of the urine during its passage through different parts of the urinary tract, which has been carried out by Richards and his co-workers [Richards, 1929, 1938] and by the intravital microscopic investigations on the frog's kidney by Ellinger & Hirt [1929*a, b, c*, 1930*a, b*, 1931] and Ellinger [1934*a, b, c*, 1935]. The evidence in favour of the secretory function is far less complete. Apart from some investigations on the excretion of dyestuffs which yielded contradictory results, it rests mainly on the classical experiments of Nussbaum on the frog's kidney [1878, 1879] and is supported by the corresponding experiment of Ghiron on the mouse's kidney [1912, 1913] and by the observation that injected acid is excreted through the epithelium of the proximal tubules of winter frogs [Ellinger & Hirt, 1930*a*].

The results of Nussbaum's experiment showing the cessation of urine formation after ligation of the renal arteries in frogs and its restoration by injection of urea has been confirmed by many workers, but interpreted in different ways. Some investigators believed that the urea

diastole, together with a slight rise in the venous pressure and fall in the circulation rate. This is the only procedure which leads to an inverse relation between them, and is possibly due to a slight incompetence in the valves. The mean arterial pressure appears to rise slightly; this may be due to the non-linear character of the flow past the narrow constriction formed by the screw-clip.

It should be noted that a reduction in the speed of the pump does *not* bring about a reduction in the output (within limits), since the longer diastolic pause allows more time for venous filling. In this respect the model differs from the mammalian circulation, except at very high heart frequencies.

SUMMARY

A circulation model is described in which the output of the pump, and hence the rate of flow round the circuit, depends upon the degree of filling, i.e. the "venous" pressure. Records are presented showing the effects of "haemorrhage", "capillary dilatation", "arteriolar constriction and dilatation", the "erect posture" and elimination of the "arterial" elasticity.

REFERENCE

Winton, F. R. [1936]. *J. Physiol.* 87, 65*P.*

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opens collaterals between the renal portal vein and the glomerular capillaries and so restores the glomerular circulation and filtration. If urea acts in this way the value of the experiment for the theory of urine formation would be restricted, while if it stimulates an excretion of urine in the proximal tubules the experiment would lend strong support to the theory of Bowman and Heidenhain. It was felt that a repetition of Nussbaum's experiment, using intravital microscopy, might decide which of the two interpretations is correct, since this method allows direct visual observation of all parts of the frog's kidney during the whole course of the experiment.

A similar re-examination might also elucidate the mechanism of Ghiron's experiment. Ghiron abolished urine formation in mice by severing the spinal cord and thus lowering the blood pressure. Urine formation was restored by injection of urea. His technique has been criticized, but his original observations have never been fully repeated.

This paper presents the results of an investigation of Nussbaum's and Ghiron's experiments by intravital microscopy and includes further observations on the elimination of certain dyes, urea and acid from the normal amphibian and mammalian kidney. The normal function of the mammalian kidney has not yet been studied by intravital microscopy. Since their experiments gave evidence for the existence of an excretory function of the proximal tubules, the question arose whether it is to be regarded as part of the normal mechanism of urine formation.

METHODS

The experiments were carried out on frogs and rats. Those on frogs with abolished glomerular function were made in May, June and July, the others at various times during three consecutive years. For the study of the elimination of dyes fluorescein and acriflavin were used. They were injected in 0.1% concentration in Ringer's solution. Urea was injected in 5% solution and acid as sodium bisulphate in 1.65% concentration. In order to examine repeated stimulation of tubular excretion two different methods were adopted. In the one the effects of either bisulphate or urea injected repeatedly at half-hourly or hourly intervals were studied on the elimination of previously injected fluorescein. In the other the excretion of fluorescein was examined after a course of daily injections, for ten to twenty days, of either bisulphate or urea.

In frogs all observations were carried out on the left kidney while dyes and drugs were injected into the lymph sac of the right leg. In rats

either of the kidneys was used for observation and all injections were made into the peritoneal cavity. The special techniques employed were as follows:

Experiments on frogs. *R. esculenta*, of both sexes, from Hungary, weighing 70–100 g. were kept cold in the dark with access to running water but without food. For intravital microscopic examination they were anaesthetized with urethane percutaneously. The abdominal vein was exposed and cut between ligatures. The abdominal muscles were incised and removed and the peritonum was opened along the lateral edge of the left kidney, which was thus exposed to the objective. The method has been described in detail by Ellinger & Hirt [1929b]. In the experiments in which the glomerular function was abolished by arterial ligation (Nussbaum's experiment), care was taken to ensure the cutting off of all possible sources of arterial supply. Under urethane the dorsal part of the portal vein was ligated and divided. In female frogs the ovaries were first removed. The kidneys were turned so as to bring the aorta and iliac arteries to the surface. Under a dissecting microscope all branches from these vessels to both kidneys as well as the mesenteric arteries were cut between ligatures. The kidney was turned back and any still pulsating branches reaching the kidney from the vesical or the ureteric arteries were looked for. Only in two of eleven experiments were such branches found. They came from the iliac artery along the ureter and were also cut. All glomeruli were then examined under the intravital microscope and re-examined an hour later. Four frogs, in which a few glomeruli were seen to be active, were discarded. In the remaining seven cases (five males, two females) all glomeruli were inactive. The circulation in the veins was not affected.

Experiments on rats. Rats of 150–200 g. of both sexes from the Lister Institute's stock were used. The surface of the rat's kidney shows only proximal and occasionally a few distal tubules. The glomeruli and all other distal tubules are situated more deeply so that, in order to expose them, the surface of the kidney must be shaved off. This operation causes serious bleeding. According to a suggestion of Hirt, it can be stopped by employing a heated knife or by applying boiling Ringer's solution. The following operative technique was developed: in a preliminary operation under ether the capsule of the exposed kidney is incised, a shaving taken from the surface with a hot scalpel, and the wound closed with silk. Two or more days later, 1 ml. of a 3% chloral hydrate solution per 100 g. rat is given intraperitoneally, the kidney is fixed on a plate with a slit for the hilum and the plate then fixed to a heated stage which allows the air round the rat to be heated to 37° C. The plate prevents respiratory movements affecting the kidney. Ringer's solution at 38–40° C. is used for irrigation.

For Ghiron's experiment rats of 200 g. weight were used. A few days after the surface of the kidney had been shaved off the rats were anaesthetized with chloral hydrate and ether. The cervical vertebrae were exposed and identified, the head was bent forward as far as possible and a fine double-edged scalpel inserted into the appropriate intervertebral space in order to cut the cord at the fifth or sixth segment. Four of ten animals survived the operation, the others dying from respiratory failure from shock which might have been avoided by adequate narcosis. The best anaesthetic is gas, but this was unfortunately not available. The wound was closed, the animal wrapped in cotton-wool and kept in a hot room at 37° C. Next day microscopic examination of the kidney revealed a very slow circulation in the intertubular capillaries. No glomeruli were found to be active in three animals, in one there was a slow circulation in a few glomerular capillaries. The glomerular capsules were empty and only a few greenish lyochromes were visible.

RESULTS

Experiments on frogs

In frogs with intact arterial supply to the kidney only a certain proportion of the glomeruli were active. Greenish fluorescent lyochromes [Ellinger, 1938] were seen only in the capsules of active glomeruli and in the lumen of tubules derived from them. Spontaneous changes in activity of the glomeruli occurred only occasionally. The elimination of fluorescein and acriflavin and the staining of the different cells of the kidney with these dyes did not vary in the experiments carried out in summer and winter. Seasonal differences in the kidney function noted in Heidelberg [Ellinger & Hirt, 1929c, 1930a, 1931] were not observed in this country.

When the arterial supply to the kidney was abolished the glomerular capsules did not contain lyochromes and none was seen in the tubular lumen.

Elimination of fluorescein. 1-2 min. after the injection of fluorescein (0.1-2.0 mg. per frog) the dye was seen in the arterioles. Almost simultaneously it appeared in the glomerular capsules with a greenish fluorescence which according to Ellinger & Hirt [1930b] corresponds to pH 7.0. 3-5 min. after injection, the lumen and the epithelium of the proximal tubules became brighter with a greenish yellow fluorescence (pH 6.5) and after another 5 min. a rather yellow fluorescence (pH 6.0) appeared in the distal tubules spreading with a slightly greener tint (pH 6.5) into the epithelium there. 15 min. after the injection a bright yellow fluorescence (pH 5.5) was seen in the collecting tubules and ureter, and in the epithelium of the former the nuclei were stained and the dye separated out in the cytoplasm. The increased fluorescence in the lumen and cells of the convoluted tubules lasted 1-3 hr. and that in the cells of the collecting tubules for about 10 hr. These changes were seen only in tubules connected with active glomeruli. Therefore the dye must have come from the glomeruli and entered the epithelium only from the lumen (Figs. 1, 2). After death of the cell the dye spread through the whole tissue.

These observations show that in frogs with intact arterial supply to the kidney the dye is eliminated in the glomeruli in about the same concentration and at the same pH as it is present in the plasma and that there is a slight concentration and acidification in the proximal and a stronger one in the distal tubules. Compared with the results obtained in Heidelberg [Ellinger & Hirt, 1929c, 1930a, 1931] concentration and acidification were less pronounced than in summer frogs and far more so than in winter frogs.

In five frogs whose arterial supply to the kidney had been abolished, injected fluorescein (0.2-1.0 mg. per frog) was seen after 2 min. in the renal portal blood and almost at the same time in the epithelium of the proximal tubules with a rather yellow fluorescence (pH 6.0). 15 min. after the injection a fainter yellowish fluorescence (pH 5.0) appeared in the lumen of the proximal tubules. The glomerular capsules and the lumen as well as the epithelium of the distal tubules remained completely unstained. This picture lasted for at least 3-4 hr.

These results indicate that a small amount of acid fluid containing dye is excreted in the proximal tubules, but that there is not sufficient excretion of water to push forward the dye into the lumen of the lower urinary tract. A similar behaviour has been observed in experiments made in Heidelberg on summer frogs with abolished arterial supply to the kidney.

Elimination of acriflavin. In frogs with intact arterial supply to the kidney the dye was eliminated in the glomeruli in low concentration and partly reabsorbed and concentrated in the tubules.

Acriflavin (0.2-2.0 mg. per frog) was seen in the blood about 5 min. after its injection. The fluorescence was not so pronounced as that observed after fluorescein injection. A little later the nuclei of the blood corpuscles of the glomerular epithelium, and of the vascular muscle cells were stained. The glomerular capsules brightened distinctly but not intensely. The lymph spaces bordering the epithelium of the proximal tubules fluoresced very brightly, those bordering the distal tubules to a much less degree. The cells were stained at first diffusely, then their nuclei took up the stain temporarily and during the next hour or two the stain was gradually aggregated into discrete granules in the cytoplasm. In the meantime the pericellular spaces had become darkened. While the lumen of the whole tubular system down to the ureter was fluorescent the epithelium of the collecting tubules was not stained as when fluorescein was injected.

Acriflavin was not so markedly concentrated in its passage downwards as fluorescein. It was seen in the lumen only of tubules derived from active glomeruli, but was contained in the epithelium of all convoluted tubules alike. This fact and the appearance of fluorescence in the pericellular lymph spaces before it reached the cells suggested that the dye had entered the cells from the lymph and blood and not from the lumen.

The fluorescence of the lumen lasted 10-24 hr., but acriflavin particles were present in the cells many days later, even after the death of the cell. The mechanism of staining of the tubular epithelium, observed in these experiments, has also been noted in experiments made in Heidelberg with acriflavin on summer frogs.

When the arterial supply to the kidney had been abolished no acriflavin was excreted.

The dye (2 mg. per frog) appeared in the blood 7-10 min. after its injection and stained the nuclei of the blood corpuscles, of the vascular muscle cells, and of the glomerular epithelium. Almost at the same time the edges of the epithelium of the proximal tubules became fluorescent and a little later the dye passed from the circumference into the interior of the cells, became diffused in the cytoplasm and stained the nuclei temporarily. About 2 hr. later it had formed large particles in the cytoplasm. No dye was seen in any portion of the lumen, a result contrary to that obtained when the glomeruli were active. Only in the proximal tubules were the cells stained.

Effect of a single injection of urea or sodium bisulphate. In frogs with intact arterial supply to the kidney, urea activated all inactive glomeruli and stimulated both mechanisms of urine formation, e.g. glomerular

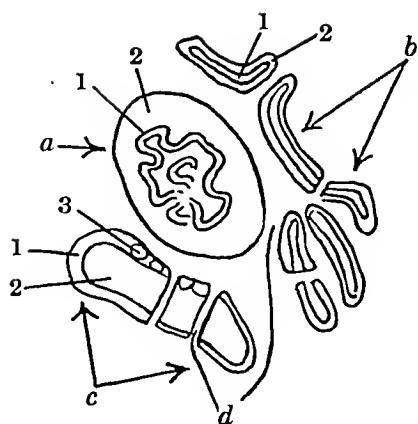


Fig. 1

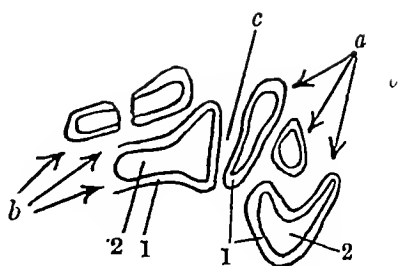


Fig. 2

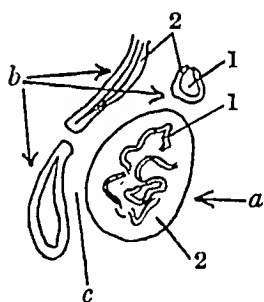


Fig. 3

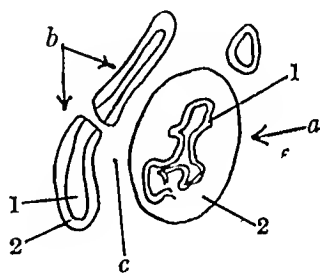


Fig. 4

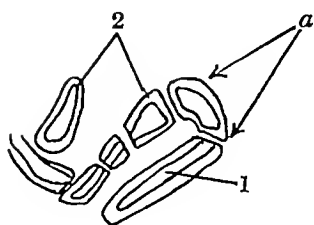


Fig. 5

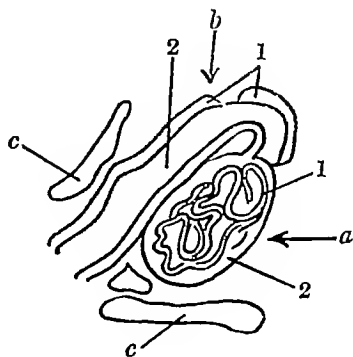


Fig. 6



Fig. 1.

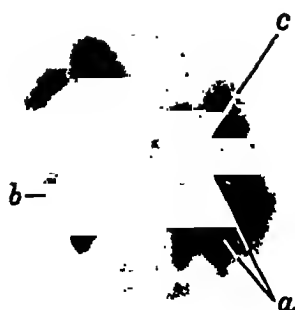


Fig. 2.

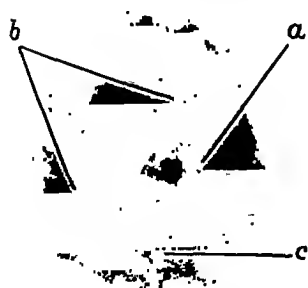


Fig. 3.

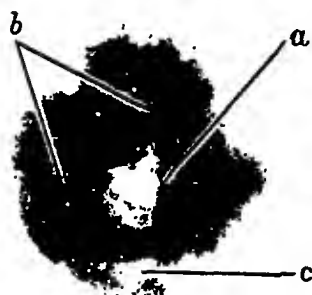


Fig. 4.



Fig. 5.



Fig. 6.



Fig. 7.



Fig. 8.

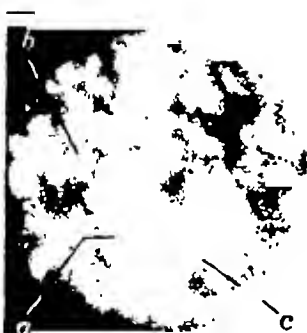


Fig. 9.

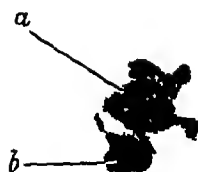


Fig. 10.



Fig. 11.



Fig. 12.

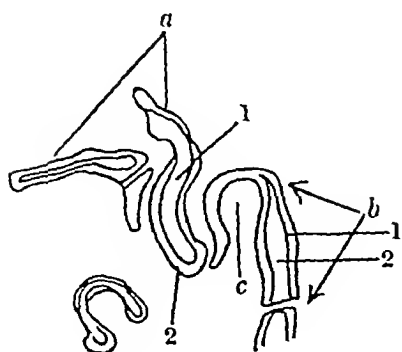


Fig. 7

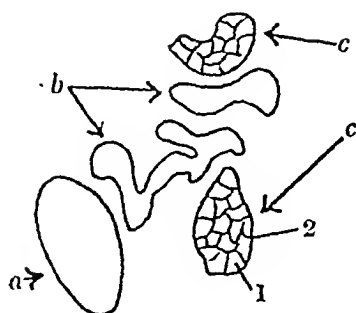


Fig. 8

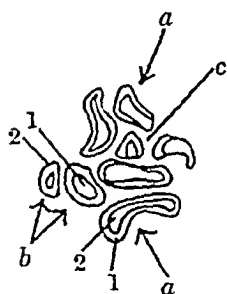


Fig. 9

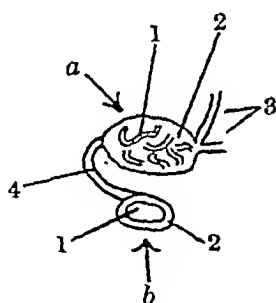


Fig. 10

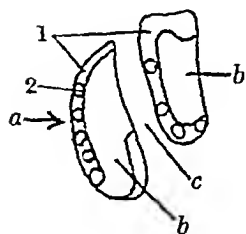


Fig. 11

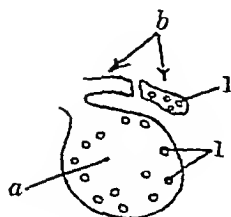


Fig. 12

filtration and tubular excretion. With bisulphate there was some glomerular filtration but the acid was mainly excreted through the proximal tubules. Similar observations have been made on winter frogs in Heidelberg [Ellinger & Hirt, 1930*a*]. The results are illustrated by the following observations.

When urea (0.25 g. per frog) was given at the height of elimination of fluorescein (2 mg. per frog previously injected), all inactive glomeruli became instantly active and the fluorescence in the lumen of the proximal tubules became a much lighter yellow (pH 5.0). This change in colour extended rapidly into the lumen of the distal tubules and beyond. The point where the greenish fluorescent fluid (pH 7.0) from the glomerular capsules met the more acid fluid in the proximal tubules could be clearly distinguished. In these experiments the dye was eliminated in 1-1½ hr. in contrast to the customary 5-6 hr. taken in the absence of urea even when only small doses of dye were injected. When a single injection of sodium bisulphate (16.5 mg. per frog) was given after fluorescein (2 mg. per frog) the fluorescence of the glomerular capsules became instantaneously lighter yellow, and at the same time still a lighter yellowish tint was seen in the epithelium of the proximal tubules and passed into their lumen. This light yellow colour moved quickly down into the lower parts of the urinary tract and after 20-30 min., the tint of the fluorescence in the whole urinary tract was as it had been before the injection of bisulphate.

In frogs with abolished arterial supply to the kidney the glomeruli were not activated by urea, but the excretion of acid urine was stimulated through the epithelium of the proximal tubules. The urine contained fluorescein but no acriflavin when either of these dyes was previously injected.

These results were obtained on five frogs in which urea (0.25 g. per frog) was injected 1-2 hr. after the dyes had been given. In the case of acriflavin the injection of urea caused no change of the staining and no dye was visible in the lumen at any time. In the case of fluorescein, immediately after the injection of urea, the epithelium of the proximal tubules became brighter with a bright yellowish fluorescence (pH 4.5) in their lumen. This light yellow colour rapidly passed into the lumen of the distal tubules and more slowly into the collecting tubules and the ureter. Their epithelium, however, remained unstained. All glomeruli remained inactive and there was no fluorescence in their capsules. 1-2 hr. after the injection of urea the fluorescence began to fade in the epithelium and lumen of the proximal tubules and later from the lumen of the tubules lower down (Figs. 3-7).

Effect of repeated injections of urea or sodium bisulphate. Repeated injections of either sodium bisulphate or urea at short intervals lead to damage of the epithelium of the proximal tubules which lose their excretory function and further injections of either of these drugs may then be fatal.

For instance, if, after fluorescein (2 mg. per frog), bisulphate (1.65 mg. per frog) is injected every hour, or half hour the tubular excretion of acid stops after the third or fourth injection and the frog soon dies. With urea the excretion through the proximal tubules fails after the sixth or seventh dose (0.25 g. per frog), but the animal may not be killed even by one or two further injections.

When the arterial supply to the glomeruli is abolished even 0.025 g. of urea causes excretion through the proximal tubules up to the fourth and fifth dose. Then excretion stops and the animal dies if a further dose is given showing that when there is no other means of elimination and tubular function is stimulated the injection even of urea may be lethal.

Daily injections of either urea or bisulphate also produce destruction of the epithelium of the proximal tubules. They lose their ability for selective absorption of water, alkali and dyes and also their excretory function.

This was revealed when fluorescein was injected. Otherwise urea appeared to be harmless, whereas daily injections of bisulphate caused local damage at the site of injection, i.e. oedema, haemorrhage, epidermolysis and paralysis of the sciatic nerve, and led usually later on to general oedema. The intravital microscopic examination of the kidneys showed a considerable reduction in the amount of green and yellow fluorescent lyochromes. The effect was especially pronounced after bisulphate. After daily injections of this substance occasionally a white bright fluorescence was seen in the epithelial cells of the proximal tubules while the intercellular lymph spaces were not fluorescent (Fig. 8). A similar white fluorescence has often been observed in glomeruli destroyed by parasites and could be attributed to a high concentration of calcium salts. The same explanation accounted for the finding in these experiments as shown by micro-calcium determination. When kidneys of frogs, after a course of daily injections of bisulphate, did not show definite signs of calcification a tendency for such form of damage could be demonstrated by the following procedure. The kidneys were removed in a sterile operation, kept for 2-4 days at 37° C. in a salt solution containing 8 mg. Ca-ions and 5 mg. P as inorganic phosphate per 100 ml. [Robison & Rosenheim, 1934] and then stained with silver nitrate or orange G; they showed marked calcification of the epithelium of the proximal tubules and sometimes of the walls of the renal arteries. This was never found in kidneys of untreated control frogs.

When fluorescein was injected into frogs which had been given daily injections of bisulphate the dye was visible within a few minutes in the blood and in the glomerular capsules with the greenish tint of pH 7.0 as seen in untreated animals. When the dye appeared in the lumen of the proximal tubules it had the same tint and concentration and remained so to the end of the experiment. In this case there was no fluorescence in the cells. The fluorescein reached the distal tubules only a little concentrated, their epithelium and that of the tubules beyond being stained as in normal frogs. The urine in the ureter showed also the greenish tint of pH 7.0, but the increased intensity of the fluorescence indicated that the dye was present in a higher concentration than in the lumen of the proximal tubules. After the daily administration of urea the subsequent injection of fluorescein showed a similar but less pronounced abnormality in the elimination of the dye. Little acidification or concentration was seen in the proximal tubules. If in such experiments the usual dose of urea was given at the height of elimination of the dye some staining of the epithelium occurred but no excretion through the epithelium into the lumen of the proximal tubules. Glomeruli previously inactive became active and the dye in the tubules was more dilute.

In three frogs which had been given daily injections of bisulphate the glomerular function was abolished by arterial ligation. The subsequent injection of fluorescein caused only slight diffuse staining of the epithelium of the proximal tubules and a little greenish fluorescence (pH 7.0) was seen in their lumen. The yellow fluorescence in the lumen of the proximal tubules which was observed in the corresponding experiments on frogs which had not undergone treatment with daily injections of bisulphate was absent. The injections of

urea caused the formation of very dilute urine containing fluorescein in about the same concentration and of the same tint as in the blood. Small amounts of bisulphate (8 mg. per frog) did not do this, larger amounts (16.5 mg. per frog) caused death.

Experiments on rats

In normal rats greenish fluorescent lyochromes were observed only in the lumen of capsules of active glomeruli and of tubules derived from them [cf. Ellinger, 1938]. A much smaller proportion of the glomeruli was active than in frogs and spontaneous changes were infrequent. In principle the reaction of the rat's kidney to injected dyes and drugs was similar to that of the frog's kidney. This was true for the experiments with intact and abolished glomerular circulation.

Elimination of fluorescein. Fluorescein (0.25–2 mg./100 g.) injected into the peritoneal cavity immediately caused the blood in the kidney to become bright and in less than 1 min. the active glomeruli showed an increased greenish fluorescence (pH 7.0) in the capsules. Almost at the same time the proximal tubules connected with them became brighter and their greenish fluorescence became yellower (pH 6.5). Their epithelium showed the same colour. The dye was here more concentrated than in the capsules and soon became more so in their epithelium than in their lumen. After 2–5 min. the dye appeared in still more concentrated form in the lumen of the distal tubules, and also, in a fainter degree, in their epithelium. This picture lasted $\frac{1}{2}$ –5 hr. according to the dose given. Then the glomerular capsules grew darker, followed by the lumen of the convoluted tubules, the epithelium of the distal, and finally of the proximal tubules (Figs. 9, 10).

These observations show that the dye is eliminated in the active glomeruli, slightly concentrated, acidified, and partly reabsorbed in the proximal tubules. In the distal tubules it is seen in a much higher concentration and with a yellower tint. The urine is, however, less acidified than in frogs.

In rats whose glomerular circulation was abolished, fluorescein (2 mg./100 g.) appeared in the renal vessels 20–30 min. after its peritoneal injection. At about the same time the lymph spaces bordering the cells of the proximal tubules became gradually brighter and from thence the dye passed into the cells. About 1 hr. later the dye was distributed diffusely throughout the cytoplasm, both here and in the cells of the distal tubules. There was none in the lumen and no dye was found in the bladder at the end of the experiment.

These results show that when the glomerular circulation is abolished no urine is formed and the tubular epithelium takes up the stain from the lymph spaces.

Elimination of acriflavin. As in frogs acriflavin stains the tubular epithelium independently of its elimination. Entering the cells from the lymph spaces the dye is eliminated in active glomeruli in low concentration and slightly concentrated during its passage through the tubules. This is shown by the following experiments.

Acriflavin (0.5–4 mg./100 g.) was not visible in the blood of the normal rat until 10–15 min. after its injection, when the nuclei of the leucocytes and of the arteriolar muscle cells

were also stained. A few minutes later the dye was seen in the capsule, in the nuclei of the glomerular epithelium and also in the lymph spaces bordering the epithelium of the proximal tubules. From the lymph spaces it entered the cells, at first staining the cytoplasm diffusely, after $\frac{1}{2}$ -3 hr. it stained temporarily the nuclei, and 2-5 hr. later it became aggregated into relatively large particles in the cytoplasm. When it was entering the epithelium from the lymph spaces the dye could also be seen coming down from the glomeruli in the lumen of the proximal tubules. Some may have been reabsorbed there, but the greater part reached the lumen of the distal tubules in but slightly greater concentration. Here the epithelium took up the stain only slowly and not intensely, either from the lumen or from the lymph or blood vessels. Only the epithelium of those distal tubules which had previously shown green fluorescence, and were connected with active glomeruli, contained the dye, whereas the epithelium of all the proximal tubules was stained. This picture lasted for many hours, even if small doses of dye had been injected (Figs. 11, 12).

In one rat with abolished glomerular circulation acriflavin (4 mg./100 g.) was seen about 30 min. after its injection in the epithelium of the proximal and later in that of the distal tubules. This picture remained unchanged for many hours and no dye could be observed in the tubular lumen or in the bladder.

There is therefore no elimination of acriflavin when the glomerular circulation is abolished.

Effects of single injections of urea or sodium bisulphate. As in frogs, a single injection of urea activated all visible inactive glomeruli and stimulated simultaneously excretion of acid urine through the epithelium of the proximal tubules. Fluorescein or acriflavin previously injected, and staining the epithelial cells of the proximal tubules, left the cells while the excretory function was working. Acid (sodium bisulphate) when injected was also at least partly excreted through the proximal tubules. These facts are revealed by the following observations.

When a large dose of urea (0.25 g./100 g.) was injected into normal rats after fluorescein (2 mg./100 g.) had been given, and its elimination had reached its height, instantly all the glomeruli in view and all their capillaries became active and charged with dye. Within a few minutes, the colour in the epithelium of the proximal tubules began to fade and their lumen as well as that of the distal tubules became bright and of a lighter yellow colour. Occasionally the lymph spaces bordering the cells would suddenly fluoresce intensely for a few seconds. In about an hour the fluorescein had all been eliminated, far quicker than in the corresponding experiments without urea. When sodium bisulphate (16.5 mg./100 g.) was injected under similar conditions the fluorescence of the capsules became more yellow and that in the lumen of the proximal tubules became suddenly very bright and assumed a pale yellow tint (pH 5.5). This colour moved down quickly into the lumen of the distal tubules and the tubules beyond. An injection of urea (0.25 g./100 g.) given during the elimination of acriflavin produced no marked changes other than activation of the glomeruli. The concentration of dye in the lumen of the tubules was not increased, and in two of the three experiments performed the epithelial cells became free of the dye presumably by way of the capillaries or lymph spaces. Occasionally these flushed brightly for a moment. Contrary to the results obtained with fluorescein the concentration of acriflavin in the urine in the bladder was lower than that found after injection of this dye without urea.

In rats with abolished glomerular circulation the injection of urea did not activate inactive glomeruli, but stimulated excretion of acid urine through the proximal tubules, containing fluorescein but no acriflavin, previously injected.

For instance, when urea (0.25 g./100 g.) was injected after the injection of fluorescein the glomeruli remained inactive, but the circulation in the capillaries of the tubules improved. No dye was seen in the capsules, but the lumen of the proximal tubules rapidly became bright yellow (pH 5.0) and the dye passed into the distal tubules. This picture remained unchanged for several hours until the death of the animal. The bladder always contained small amounts of stained urine. Even injections of 0.025 g. of urea per 100 g. caused an excretion of urine through the proximal tubules. After acriflavin (4 mg./100 g.) the injection of urea (0.25 g./100 g.) caused no change of the staining and no acriflavin was seen in either tubules or bladder.

Effects of repeated injections of urea or sodium bisulphate. The results obtained on rats with normal as well as with abolished glomerular circulation are similar to those obtained in frogs.

When, after fluorescein, sodium bisulphate (8-16.5 mg./100 g.) was injected every half hour or hour, excretion of acid urine through the epithelium of the proximal tubules was obtained only after the first two or at most the first three injections. The rats may die in coma even after the second injection. The yellowish fluorescence which was observed under similar conditions with a single injection of urea can still be obtained when urea (0.1 g./100 g.) is repeatedly injected up to the fifth or sixth injection, but not with later ones although even ten injections given at intervals of $\frac{1}{2}$ or 1 hr. may be survived.

In rats with abolished glomerular circulation excretion through the proximal tubules can be stimulated by injections of urea (0.025 g./100 g.) repeated every half an hour or every hour up to the fourth or fifth injection. Then the excretion fails and the rats die if the injection is continued.

This repeated stimulation of tubular excretion at short intervals annihilates this function and even urea becomes toxic if tubular excretion is abolished and no other means of elimination are available.

Daily injections of urea in doses up to 0.25 g./100 g. did not produce any toxic effect but sodium bisulphate given daily in small doses (6-8 mg./100 g.) for 10-20 days or in larger doses (16.5 mg./100 g.) for 2-3 days produced coma and death. At autopsy local inflammation was present in the intestines. As in frogs the number of green and yellow lyochromes in the kidney was considerably reduced, particularly after bisulphate. After a course of daily injections of urea or bisulphate the injected fluorescein appeared within a few minutes in the capsules and passed into the proximal tubules in about the same concentration and colour (pH 7.0) without staining the epithelium, and into the distal tubules in somewhat greater concentration but still of the same tint. As in corresponding experiments in frogs the reabsorption of water, dye and alkali in the proximal tubules and the staining of the epithelium had ceased, but the reabsorption of water in the loops of Henle was not affected. A subsequent injection of urea caused no marked change, but small doses of bisulphate (8 mg./100 g.) temporarily changed the fluorescence in the capsules and in the lumen throughout the tubules to a yellow tint, showing that glomerular filtration of acid was also not affected. Larger doses of bisulphate killed the animal.

In two rats in which after a course of bisulphate the cord was cut, urine formation stopped, and after an injection of urea (25 mg./100 g.) fluorescein previously injected was seen neither in the capsules nor in the proximal tubules, though the epithelium showed sometimes a faint green fluorescence. Small amounts of bisulphate (up to 8 mg./100 g.) injected under similar conditions had no effect. Larger ones killed the rat.

These experiments give evidence that daily injections of bisulphate destroy also the excretory function of the proximal tubules. The occasional staining of the epithelial cells with fluorescein of the same tint and concentration as in the blood, caused by diffusion of the dye into the cells, shows also the loss of the selective permeability of the cell membranes.

DISCUSSION

The absence in the present experiments of seasonal differences in the function of the frog's kidney illustrates that results obtained in different countries or on different species of frogs may vary to a great extent. In England, in all seasons, most of the partial functions of the kidney behaved in a manner intermediate between that shown by summer and winter frogs in Heidelberg, but more similar to that of the former. This was true for the mode of staining of the tubular epithelium with fluorescein and acriflavin, for the pH of this epithelium and of the preliminary urine in the lumen of the tubules, and for the finding that only a comparatively small proportion of the glomeruli was active and that their activity seldom altered spontaneously. The response of the kidney with abolished arterial supply to fluorescein was equal to that obtained in similar experiments during summer in Heidelberg, while the excretion of acid urine through the proximal tubules after the injection of bisulphate corresponded to the response obtained during winter in Germany. Seasonal differences or the use of different species of frogs may also account for the conflicting evidence concerning the site of acidification. Montgomery & Pierce [1937] found that in American frogs, *R. pipiens*, acidification of the urine occurred only in certain parts of the distal tubules. In the present experiments carried out in this country on Hungarian frogs, *R. esculenta*, there was a definite acidification in the proximal as well as in the distal tubules, the effect being stronger in the latter. Ellinger & Hirt [1929c, 1930a, 1931], however, working in Heidelberg on German frogs, *R. esculenta*, found that while in summer acidification was not only stronger but also more pronounced in the proximal than in the distal tubules, in winter the urine of these frogs was on the whole much less acidified.

The observation on the frog's kidney that acriflavin first stained the pericellular lymph spaces of the proximal tubules, whether their glomeruli were active or not, and later stained the cells, shows that this dye reaches them from the blood or lymph and not from the lumen. A similar phenomenon was observed by Ellinger & Lambrechts [1937] using rhodamin B. The fact that none of the acriflavin passed from the cell into the lumen illustrates that the presence of a dye in the epithelium does not necessarily imply that it is being excreted there. The taking up of an injected dye by the tubular epithelium and its excretion are processes independent of one another and its absence in the lumen when it is present in the epithelial cells is no evidence in favour of the theory of Bowman & Heidenhain. Singer [1933], who repeated the experiments of Ellinger & Hirt on American *R. pipiens* with results in general agreement with ours, believed that he observed injected acriflavin in the tubular lumen before it appeared in the glomerular capsules. He injected acriflavin only when the elimination of injected aesculin was at its height. Under such conditions it is not possible to decide whether acriflavin stains the epithelium of the tubules alone or their lumen as well, as is often observed when acriflavin is injected after fluorescein. Acriflavin unlike fluorescein is found in a lower concentration in the capsules than in the blood. This might be due to the fact that acriflavin forms comparatively stable compounds with the blood protein which cannot be filtered in the glomerulus. The elimination of acriflavin in the urine is also far slower than that of fluorescein, since the former is partly fixed in the tissues.

Fluorescein and acriflavin are dealt with in the rat's kidney just as in the frog. Fluorescein is filtered in the glomeruli and concentrated in the proximal tubules, where water, some alkali, and a little of the dye are reabsorbed. The concentration of the dye in the distal tubules is not much higher, perhaps because some is absorbed with water in the loops of Henle which cannot be seen, as well as in the distal tubules themselves, but the urine is more acid there. Acriflavin in low concentration enters the capsules, stains the nuclei of the capsular epithelium and of the cells of the blood vessels, and enters the epithelial cells of the proximal tubules from the lymph spaces. This staining of the pericellular lymph spaces indicates that lymph takes some part in the formation of urine [cf. Keller, 1933] especially in the absence of glomerular circulation [cf. also Richards & Barnwell, 1927].

The present experiments give new evidence for an excretory function of the convoluted tubules. Acid urine was shown to be formed in the

proximal tubules of the normal kidney of frogs and rats when urea or sodium bisulphate was injected. A similar response was obtained in the experiments with abolished glomerular circulation. The intravital microscopic study completely confirmed Nussbaum's observation and the objections raised against his interpretation could be refuted. No urine was formed when the arteries were tied, but on injection of urea acid urine was formed containing, after these dyes had been injected, fluorescein but not acriflavin. These two dyes therefore correspond in their behaviour to sulphindigotate and carmine, respectively, in Nussbaum's experiment. There is no doubt that collateral circulation from the renal portal vein or genital, vesical, or ureteric arteries may be opened up by increased pressure or hypertonic urea injections, and glomerular filtration may so be re-established as in the experiments of Bieter & Hirschfelder [1922, 1924, 1926, 1929], Richards & Walker [1927], Bainbridge, Collins & Menzies [1913, 1914] and Kempton [1937]; while the injection of urea into frogs with intact arterial supply to the kidney quickly activates quiescent glomeruli as shown also by Richards [1922] and Bieter & Hirschfelder [1924]. Nothing of the kind occurred in the present experiments in which the glomerular action was directly observed. Nor can restored glomerular activity explain the results of Nussbaum [1878, 1879, 1886]. Halsey [1902], Bainbridge & Beddard [1906*a*, *b*], Tamura, Miyamura, Fakuda, Hosoya, Kishi & Kihara [1927] or Tamura, Miyamura, Nagasawa, Hosoya, Kishi & Fushita [1927], for in these, as in those now reported and in those of Ellinger & Hirt [1930*a*] on winter frogs. the urine differed from the normal in acidity and in the lack of certain injected dyes. According to Beddard [1902] the activating effect of urea can only be observed when ligation of the arteries has preceded the injection of urea sufficiently long to allow degeneration of the epithelium to occur. This objection is excluded in the present experiments in which there was no time available for degeneration. The only explanation is that, instead of glomerular filtration followed by partial reabsorption in the tubules, a different mechanism of excretion through the proximal tubules is brought into play and water and fluorescein can in fact be seen being discharged from the proximal tubules. The present results differ from those recently obtained by Kempton who observed no formation of urine if all collaterals were tied and urea was injected. It is possible that this discrepancy is again the result of seasonal or climatic variations, frequently referred to. In fact, Ellinger & Hirt [1930*a*, 1931] found that after arterial ligation spontaneous excretion of urine rarely occurred in the summer, whereas

it was regularly observed in winter frogs. In experiments of the type recorded in this paper, the urine containing acid and fluorescein but no acriflavin unquestionably was excreted through the cells of the proximal tubules.

Ghiron's experiment was also confirmed by our method. The low blood pressure abolished glomerular circulation and formation of urine, as well as elimination of fluorescein or acriflavin, although both were to be seen in the tubular epithelium. After injection of urea, the elimination of urine started again, but acriflavin still was not excreted. Fluorescein, however, was excreted through the epithelium of the proximal tubules and not through the glomeruli, which remained inactive.

The results give some indication of the role of the excretory function of the tubules. The epithelium of the proximal convoluted tubules, if its excretory function is repeatedly called into play, loses its capacity for reabsorption, for excretion, and for being stained by the dyes used. If frogs are repeatedly treated with urea or bisulphate and then injected with fluorescein and urea after ligation of the arteries, the urine formed in the tubules contains fluorescein in a similar concentration and of the same pH as in the plasma. Hence it is evident that the power of selective excretion has also been lost. Further evidence of the damage suffered is given by the increased tendency of the epithelium to take up calcium. Excretion through these cells cannot, therefore, be a constant function. It may come into action in emergency or in pathological conditions when the glomeruli fail or the body is loaded with substances which must be removed quickly. This does not exclude the possibility that under normal conditions some urine constituents may be partly eliminated through the proximal tubules in a concentration more or less similar to that present in blood. Such an elimination would not involve great osmotic work which apparently damages the cells concerned, and could be responsible for the tubular excretion of phenol red postulated by Marshall & Vickers [1923] and Marshall [1931] by an indirect method. The studies by Walker & Hudson [1937 *a, b*] of a possible tubular excretion of urea and phosphate, which would also represent such a type of elimination, did not lead to conclusive evidence.

The epithelium of the proximal tubules may act in two ways: in normal conditions by absorbing water or certain solutes from the glomerular filtrate and perhaps by elimination of certain constituents in comparatively low concentration, and in abnormal conditions by excreting water, solids, and acid from the blood. The way in which it acts will depend on the concentration gradient of substances to be absorbed

or excreted, as between the epithelium and lumen on the one hand and the blood and the lymph on the other. What substances are absorbed or excreted apparently depends on a selective one-way permeability of the cell walls bordering the lumen.

This conception combines some features of the view of Ludwig and Cushny with others of the theory of Bowman and Heidenhain, the normal interplay of filtration and reabsorption being supplemented in special circumstances by an excretory function of the proximal tubules. This extension of Ludwig and Cushny's theory may possibly explain some pathological conditions, e.g. the secondary nephrosis following acute or chronic glomerulitis, and the constitution of the urine in acute glomerulonephritis.

SUMMARY

1. The intravital microscopic study of the response of the normal frog's kidney to injected fluorescein and acriflavin confirms generally the experiments by Ellinger & Hirt and reveals

(a) that seasonal variations of the behaviour of the frog's kidney observed in Heidelberg did not occur in this country;

(b) that acriflavin stained the epithelium of the proximal tubules independently of the elimination of the dye, entering the cells from the intracellular lymph spaces;

(c) that acriflavin, contrary to fluorescein, is eliminated in the glomerular capsules in a lower concentration than it is present in the blood.

2. In the normal rat's kidney both dyes were eliminated in a manner similar to that in the frog.

3. In normal frogs and rats a single injection of a large dose of either urea or sodium bisulphate stimulates the excretion of acid urine through the epithelium of the proximal tubules.

4. The study here made, by intravital microscopy, of Nussbaum's and Ghiron's experiment confirms the original observations and interpretations of these workers: ligation of the arteries of the frog's kidney or reducing the blood pressure by severing the spinal cord in rats, arrests the glomerular function and the formation of urine in both cases; while after injection of urea, urine different from the normal urine is excreted in the proximal tubules.

5. Repeated stimulation of the excretory function of the proximal tubules leads to pathological changes in the cells concerned. This excretory function, therefore, cannot be regarded as normal; rather must

it be regarded as an emergency function in the absence of glomerular filtration or when the blood is overloaded with substances requiring quick elimination.

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EXPLANATION OF PLATES I AND II

Figs. 1-12. Photographs of living kidneys (magnification: Figs. 1-10 and 12 $\times 78$, Fig. 11 $\times 150$. Zeiss Miflex, reducing the magnification to $\frac{1}{2}$. Ilford Panchromatic Plates. Eyepiece: photo $\times 4$ (Zeiss). Objective: Figs. 1-10 and 12, W.I. 39, num. Ap. 1.10; Fig. 11, E.-H. 75, num. Ap. 1.15; both special lenses for intravital microscopy (Zeiss). Filters (Schott): BG3, 2.5 and 3.5 mm., resp. and GG5, 1 mm.). The photographs are taken directly of the living organs. While these show only one level giving an average stage of the process, in the experiment the micrometer screw is constantly used to observe different levels of the object continuously. The different parts of the kidney were identified by following up the elimination of fluorescein from the glomerulus downwards and by comparing the intravital microscopic pictures with those of ordinary microscopic slides of sections.

PLATE I

- Fig. 1. Frog, kidney, ventral side; 2 hr. 51 min. after injection of 0.25 mg. fluorescein: oval active glomerulus (a) with capillaries (a_1) and bright capsule (a_2), some distal tubules (b) with bright narrow lumen (b_1) and fairly bright narrow epithelium (b_2), one collecting tubule (c) with bright epithelium (c_1) and bright lumen (c_2); some of the nuclei of the epithelial cells are stained (c_3); the darker intervals between the tubules and sometimes crossing them are capillaries of the renal portal veins (d). Exp. 15 sec.
- Fig. 2. Frog, kidney, dorsal side; 21 min. after injection of 0.25 mg. fluorescein: proximal tubules, some with bright epithelium (a_1) and bright lumen (a_2) (active systems), some tubules whose epithelium is stained with yellowish fluorescent lyochrome particles (b_1), the lumen being dark (b_2) (inactive systems). Epithelium and lumen are broader than those of the distal tubules in Fig. 1. The comparatively dark intervals between the tubules are capillaries of the renal portal vein (c). Exp. 1 min.
- Fig. 3. Frog, kidney, ventral side, immediately after ligating and cutting the renal arteries: oval inactive glomerulus (a) with capillaries (a_1) (far less distinct than those in the active glomerulus, Fig. 1) and dark capsule (a_2), some distal tubules (b) with greenish fluorescent lyochromes in low concentration in the lumen (b_1) and dark epithelium (b_2), dark intertubular capillaries (c). Exp. 3 min.
- Fig. 4. Same frog, same part of the kidney, 10 min. after injection of 0.2 mg. fluorescein: no alteration of the picture. Letters mark the same parts of the picture as in Fig. 3. Exp. 3 min.

Fig. 5. Same frog as in Fig. 3, dorsal side, 13 min. after Fig. 4 (23 min. after injection of fluorescein): proximal tubules (a), low concentration of lyochromes in the lumen (a_1) and in the epithelium (a_2). Exp. 5 min.

Fig. 6. Same frog as in Fig. 3, ventral side, 2 hr. after fluorescein injection and 6 min. after injection of 5 ml. 5% urea: oval inactive glomerulus (a) with dark capillaries (a_1) and dark capsule (a_2) and one proximal tubule (b) with very bright yellow broad epithelium (b_1) and bright yellow lumen (b_2) and some dark distal tubules (c). Exp. 1 min. 15 sec.

PLATE II

Fig. 7. Same frog as in Fig. 3, ventral side, 2 hr. 30 min. after injection of fluorescein and 36 min. after urea injection: distal tubules (a) with bright yellow narrow lumen (a_1) and bright narrow epithelium (a_2), one distal tubule with fairly bright epithelium (b) and dark lumen (b_1), fairly bright intratubular capillaries (c). Exp. 45 sec.

Fig. 8. Frog, injected 12 times with 1 ml. 1.65% sodium bisulphate in 17 days: kidney, ventral side, dark glomerulus (a), some distal tubules (b) with greenish fluorescent lyochromes in the lumen and two proximal tubules (c) with very bright fluorescent calcified epithelial cells (c_1) and darker cell edges (c_2). Exp. 3 min.

Fig. 9. Rat, kidney, surface; 25 min. after injection of 3 mg. fluorescein: proximal tubules, some with bright lumen (a_1) and bright epithelium (a_2) (active systems), some with dark lumen (b_1) and fairly bright epithelium (b_2) (inactive systems). Blood capillaries (c) between the tubules, sometimes crossing them. Exp. 1 min.

Fig. 10. Rat, kidney, after the surface had been shaved off; 3 hr. 20 min. after injection of 3 mg. fluorescein: active glomerulus (a) with fairly dark capillaries (a_1), bright capsule (a_2), vas afferens and efferens (a_3) and neck leading to a proximal tube (a_4), and a distal tube (b) with very bright lumen (b_1) and bright epithelium (b_2). Exp. 2 min.

Fig. 11. Rat, kidney, surface; 2 hr. after injection of 3 mg. acriflavin: proximal tubules, fairly bright epithelium (a_1) with very brightly stained nuclei (a_2) and fairly dark lumen (b). Between the tubules the dark capillaries (c). Exp. 20 sec.

Fig. 12. Rat, kidney, after the surface had been shaved off; 3 hr. 15 min. after injection of 3 mg. acriflavin. Active glomerulus (a), the nuclei of the glomerular epithelium being stained (a_1), and vas afferens (b) with stained nuclei of the vascular muscle cells (b_1). Exp. 1 min. 30 sec.

THE ACTION OF SOME AMINES RELATED TO ADRENALINE. CYCLOHEXYLALKYLAMINES

By J. A. GUNN AND M. R. GURD

From the Nuffield Institute for Medical Research, Oxford

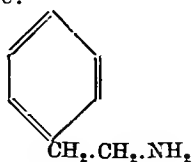
(Received 29 July 1939)

In previous papers of this series the actions of a number of compounds related to adrenaline have been described and a summary of their actions was given in a recent paper [Gunn, Gurd & Sachs, 1939]. All of the compounds previously dealt with have a phenylethylamine or phenylisopropylamine skeleton and most of them possess one or more methoxyl groups, or a methylenedioxy group, on the benzene ring. A wider survey of compounds related to adrenaline has recently been made by one of us in the Oliver-Sharpey Lectures [Gunn, 1939], in the course of which some conclusions were drawn relating physiological action and chemical constitution within this group of compounds.

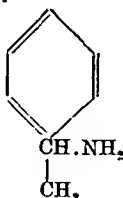
The present paper deals with the actions of a series of amines having a partially or completely reduced ring, and a methylamine, ethylamine, or isopropylamine side-chain. In view of the large number of compounds related to adrenaline that have been synthesized and investigated, it is surprising that so little attention has been paid to the effects of reduction of the ring, though this has considerable theoretical interest as well as providing a new series of compounds with potential therapeutic values. For purposes of comparison we have included experiments also with α - and β -phenylethylamines. Several references occur in the literature to the action of the latter compound which will be mentioned in the discussion at the end of the paper. To facilitate comparison with previous compounds in the series, the compounds here discussed have been numbered consecutively with those in the last paper, and the pharmacological actions are dealt with in the same order.

The following are the compounds to be considered:

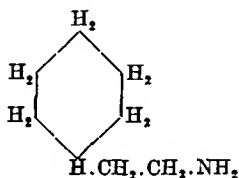
(14) β -Phenylethylamine:



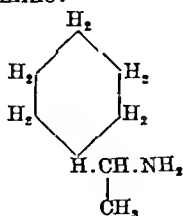
(15) α -Phenylethylamine:



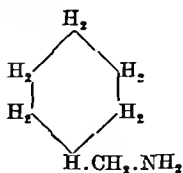
(16) β -Cyclohexylethylamine:



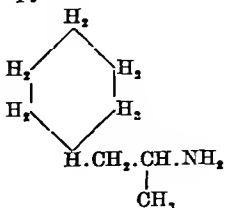
(17) α -Cyclohexylethylamine:

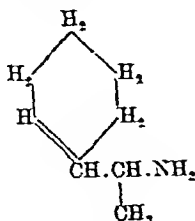


(18) Cyclohexylmethylamine:



(19) β -Cyclohexylisopropylamine:



(20) β . Δ .1.1.2.Cyclohexenylisopropylamine:

The first five compounds were available as hydrochlorides, the last two as sulphates, and the doses refer to these salts.

RESULTS

Toxicity

The toxicities of these compounds were determined in white mice by intraperitoneal injection. Preliminary estimations were made by injection of graded doses, not less than ten animals being used for each compound. Subsequently the L.D. 50 was determined on at least two groups of twenty mice each. The following table gives the resulting L.D. 50 for all the compounds.

Compound	14	15	16	17	18	19	20
L.D. 50 (mice, intraperitoneal)	(g./kg.)	0.23	0.26	0.12	0.18	0.36	0.16	0.09			

These figures bring out some points of interest. Reduction of the ring increases the toxicity, for β -cyclohexylethylamine (16) has about twice the toxicity of β -phenylethylamine (14), and α -cyclohexylethylamine (17) about one and a half times the toxicity of α -phenylethylamine (15). The β compounds are slightly more toxic than the α compounds both in the case of phenylethylamine (14, 15) and cyclohexylethylamine (16, 17). With the cyclohexyl nucleus, the methylamine compound (18) has only about one-third of the toxicity of the ethylamine compound (16), which latter is also slightly more toxic than the isopropylamine compound (19). With previous compounds which we have investigated, it has always been found that with any given ring structure the compound with an isopropylamine side-chain is considerably more toxic than one with an ethylamine side-chain; evidently, however, this is not true of the cyclohexyl ring. Finally cyclohexenylisopropylamine (20) is about twice as toxic as cyclohexylisopropylamine (19), although the only chemical difference is a single unsaturated linkage in the ring in the former. So far as we know, this is the first compound with this particular ring structure that has been investigated in this group. It will be shown later that it is also the most powerful cerebral excitant of all the compounds we have investigated.

Symptoms produced

The symptoms produced by these compounds when injected intraperitoneally in mice were studied by simple observation and by cinematographic records. A detailed description of the symptoms produced by individual compounds would require lengthy protocols and only a brief comparative description will be given. All compounds produced symptoms of excitation of the central nervous system, characterized by frequent and rapid co-ordinated movements, accelerated respiration and sometimes tremor and ataxia. Especially with rapidly fatal doses, terminal asphyxial convulsions occurred, while in some cases especially with large but non-fatal doses a stage of partial motor paralysis followed the period of excitement. The symptoms of excitation were not identical with different compounds, and were least marked with α -phenylethylamine. In the cyclohexyl group the methylamine (18), ethylamine (16) and isopropylamine (19) compounds showed progressively increasing central stimulation in that order. When compounds with corresponding side-chains are compared, the cyclohexyl nucleus seems to be slightly more stimulant than the phenyl nucleus. Cyclohexenylisopropylamine (20) produced the most pronounced increase in central excitability of all the compounds. This is no doubt partly due to the superior stimulant properties of the isopropylamine, as compared with the ethylamine side-chain, but must be also due to the partially reduced nucleus, because it appears to be definitely more stimulant than either β -phenylisopropylamine or β -cyclohexylisopropylamine.

To compare it with the former (benzedrine) we used an arrangement for cinematographic records which may be useful to others for similar investigations. A deep wooden tray, 14 in. sq., was divided by two partitions into three compartments (Fig. 1). The side of the tray facing the camera was sloped at an angle of about 45° and, as the lens of the camera was directed at about the same angle, all the animals in the tray were permanently in the field. In one compartment (*N*) were six control mice, in (*B*) six mice injected with benzedrine and in (*C*) six mice injected with cyclohexenylisopropylamine.

The tray was covered with a sheet of plate glass, without which the animals would sometimes leap out of the tray during the excitement stage produced by the drugs. By this arrangement two compounds can conveniently be compared with one another in regard to the symptoms they produce, with the behaviour of normal mice under similar conditions as a control.

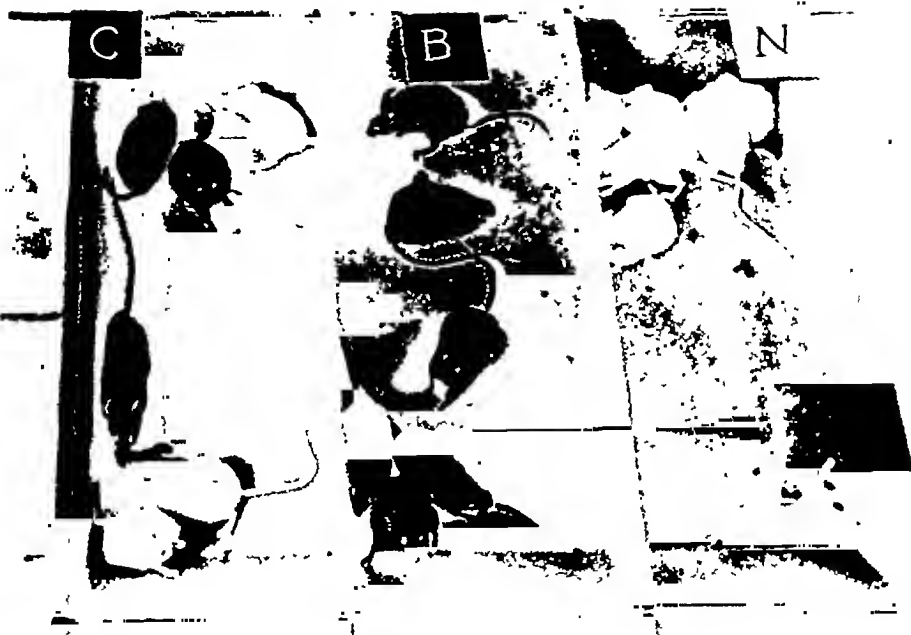


Fig. 1. Arrangement for cinematographic records (see text) permitting comparison of three groups of mice, and giving an impression of the superior activity and peculiar postures of mice injected with cyclohexenylisopropylamine (C) and with benzedrine (B) over normal mice (N).

We have found with benzedrine and allied compounds which produce a similar stimulation of the central nervous system that the symptoms of excitement are much more pronounced if several injected animals are kept together in a cage or tray. If one animal is kept alone in a jar, no very striking symptoms of excitement may be exhibited, whereas when several are together and especially if they have room to run, they excite one another. The symptoms of intoxication are very characteristic; the animals are extremely restless, make frequent rapid rushes often accompanied by squealing, and sometimes sit up on their hind quarters with inquisitive rotational movements of the head and neck. Occasionally there are clonic convulsive movements. These symptoms can be easily studied in a cinematograph film but the photograph reproduced may perhaps convey an impression of the restlessness and characteristic postures of the injected animals (in *C* and *B*) as compared with the sedentary behaviour of the control mice (in *N*). Study of the cinematographic records confirmed what had been inferred from simple observation of the animals that cyclohexenylisopropylamine produced a slightly more intense, but less enduring, motor excitement than benzedrine.

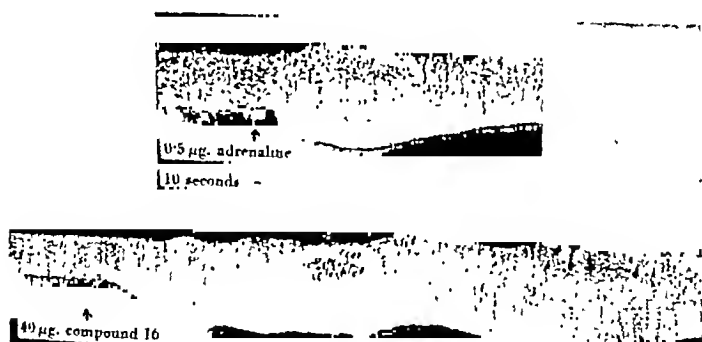


Fig. 2. Isolated perfused cat's heart. Showing more prolonged stimulant effect of 40 μ g. β -cyclohexylethylamine (lower tracing) than of 0.5 μ g. adrenaline (upper tracing).

Isolated mammalian heart

In the isolated perfused heart of the cat, all the compounds produced, in various degrees, augmentation and acceleration of the contractions.

Compounds 14 and 16 were approximately equal in activity, producing an effect about equal in excursion to that produced by one-hundredth the amount of adrenaline but much more prolonged. Fig. 2

shows the transient effect produced by $0.5\mu\text{g.}$ of adrenaline injected into the cannula immediately above the heart, compared with the prolonged effect of $40\mu\text{g.}$ of compound 16.

The α compounds 15 and 17 were also about equal in potency but their activity was much less than the corresponding β compounds 14 and 16. Compounds 18, 19 and 20 had an activity approximately equal to that of one two-hundredth the amount of adrenaline. The action of compound 20 was especially quick in onset and of short duration.

Blood pressure

The effects of the compounds on blood pressure were examined in spinal cats and in rabbits anaesthetized with urethane or ether. Quantitative comparisons of the compounds with one another and with adrenaline are only approximate.

Cats. All seven substances produced rises in blood pressure in spinal cats when injected intravenously in doses of 1–5 mg. Compound 14 was the most potent pressor substance, having from one-fiftieth to one-hundredth of the pressor activity of adrenalinic. Its action was similar to that of adrenaline, the duration of the rise being not more than twice that of an equipressor dose of adrenaline; the pressor activity did not diminish with successive doses. Compound 16 had about one-quarter of the activity of compound 14 but the pressor effect was slightly more prolonged. The corresponding α compounds (15 and 17) had much less pressor activity than the β compounds (14 and 16), compound 15 having about one-fiftieth of the activity of compound 14 and compound 17 one-seventh of the activity of compound 16.

These differences are illustrated in Fig. 3. For subsequent comparisons injections were first given of 1, 2, 4, and $8\mu\text{g.}$ of adrenaline. The tracings show the superior pressor potency of β -phenylethylamine (E) over α -phenylethylamine (F), and of β -cyclohexylethylamine (G) over α -cyclohexylethylamine (H). The injection of these compounds did not reduce the sensitivity of the animal to adrenaline, $4\mu\text{g.}$ of which produced even a greater effect after the compounds were administered than before.

Fig. 4 shows a comparison of the pressor actions of compounds 16 (A), 18 (B), 19 (C), and 20 (D), each of them given in a dose of 1 mg., which produced rises of pressure of 70, 24, 70, and 76 mm. Hg respectively. In regard to intensity, the three compounds cyclohexylethylamine (A), cyclohexylisopropylamine (C) and cyclohexenylisopropylamine (D) are about equal but the duration in B and D is about three times as long as in A. The pressor effect of cyclohexylmethylamine is

relatively feeble. In these pressor differences between the methylamine, ethylamine and isopropylamine side-chains the cyclohexyl compounds show a close similarity to the corresponding phenyl compounds.

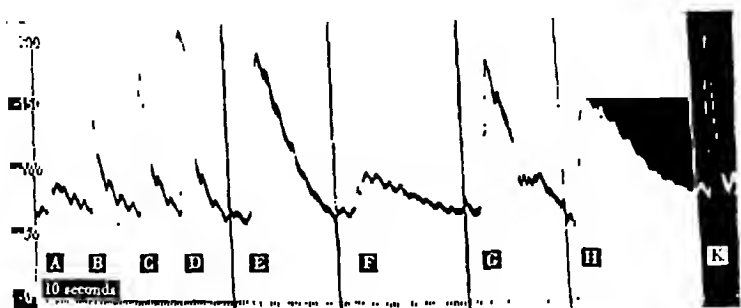


Fig. 3. Blood pressure of spinal cat. A, 1 μ g. adrenaline; B, 2 μ g. adrenaline; C, 4 μ g. adrenaline; D, 8 μ g. adrenaline; E, 0.4 mg. β -phenylethylamine; F, 3 mg. α -phenylethylamine; G, 1 mg. β -cyclohexylethylamine; H, 5 mg. α -cyclohexylethylamine; K, 4 μ g. adrenaline (at end of experiment). Showing 1 mg. β -cyclohexylethylamine equal to about 0.4 mg. β -phenylethylamine and about 6 μ g. adrenaline, and the much feebler pressor activity of the α compounds.

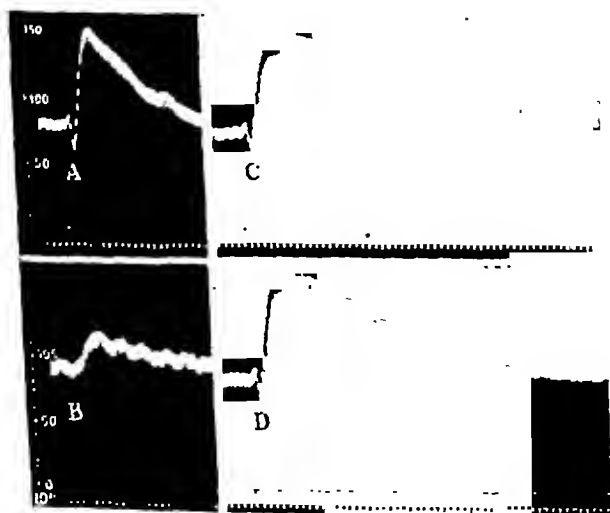


Fig. 4. Blood pressure of spinal cat. Showing effect of 1 mg. each of cyclohexylethylamine (A), cyclohexylmethylamine (B), cyclohexylisopropylamine (C) and cyclohexenylisopropylamine (D). The rises of blood pressure are about equal in A, C and D; the duration of C and D about three times that of A. The pressor activity of B is relatively feeble.

The actions of ergotoxine and cocaine on pressor effects was investigated only in the case of β -phenylethylamine and β -cyclohexylethylamine. Fig. 5 shows the effects of 1 mg. β -phenylethylamine, 4 mg. β -cyclohexylethylamine and 20 μ g. adrenaline before and after ergotoxine. The pressor action of adrenaline was reversed but that of the other two compounds merely reduced.

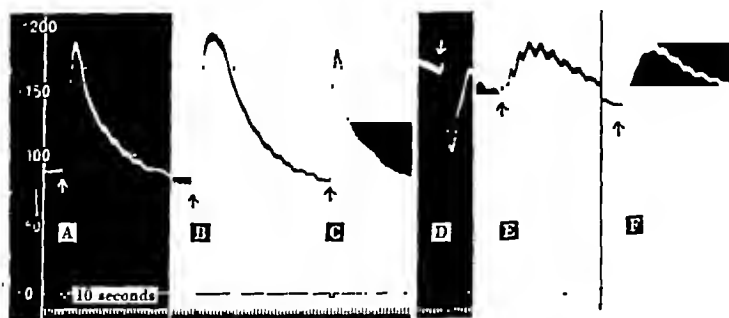


Fig. 5. Blood pressure of spinal cat. A and E, 1 mg. β -phenylethylamine; B and F, 4 mg. β -cyclohexylethylamine; C and D, 20 μ g. adrenaline. Between C and D, 0.5 mg. ergotoxine ethanesulphonate. Showing that after ergotoxine the pressor action of adrenaline is reversed but that of β -phenylethylamine and of β -cyclohexylethylamine is only reduced.

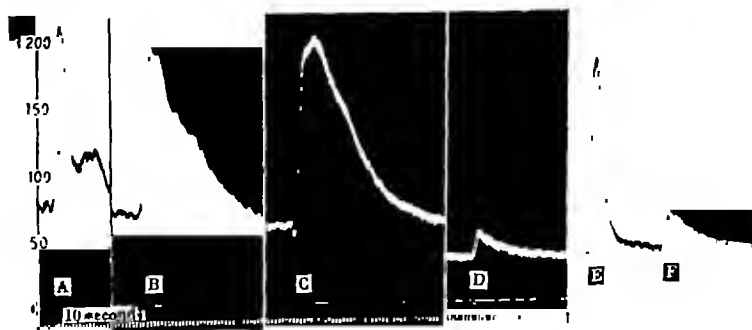


Fig. 6. Blood pressure of spinal cat. A and E, 20 μ g. adrenaline; B and D, 1 mg. β -phenylethylamine; C and F, 4 mg. β -cyclohexylethylamine. Between C and D, 15 mg. of cocaine hydrochloride intravenously. Showing that after cocaine the pressor effect of adrenaline is increased but that of β -phenylethylamine and β -cyclohexylethylamine almost abolished.

Fig. 6 shows the effect of the same doses of the same compounds before and after cocaine. Cocaine enhances the pressor effect of adrenaline and markedly diminishes the pressor effects of β -phenylethylamine

and β -cyclohexylethylamine. These results agree with what has been found with the majority of compounds related to adrenaline. They differ from adrenaline in the alteration in pressor responses produced by ergotoxine and cocaine. The pressor effects after ergotoxine are reduced but not reversed as occurs with adrenaline, and the pressor effects after cocaine are reduced whereas with adrenaline they are augmented.

Rabbits. In rabbits anaesthetized with urethane, these compounds produced a small rise of blood pressure when given in small doses (1-5 mg.) but, even after section of both vagi, the rises were much smaller

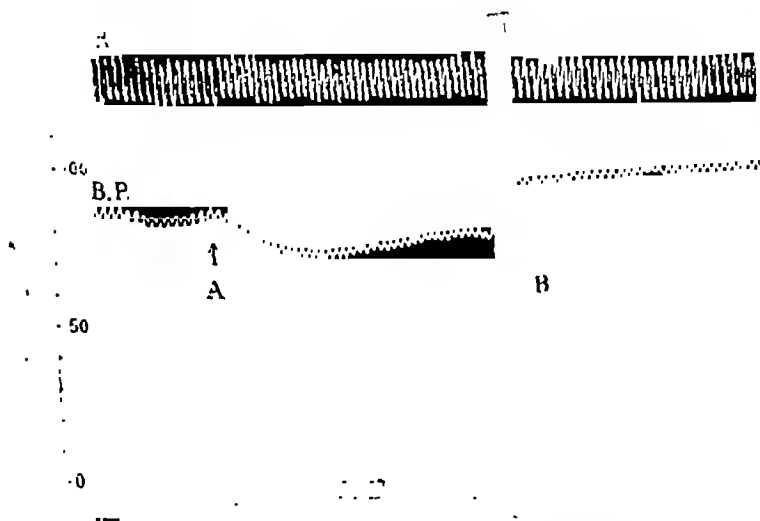


Fig. 7. Respiration (*R*) and blood pressure (*B.P.*) in a rabbit anaesthetized with urethane. At *A*, 4 mg. β -cyclohexylethylamine. Showing slight acceleration of respiration and preliminary fall of blood pressure, followed by a rise at *B* 3 min. later. Contrast the slight effects on blood pressure in the rabbit with the marked pressor effects of the same dose in spinal cats in Figs. 4*B* and 5*C*.

than were produced by corresponding doses in spinal cats, as has previously been found with other substances of the group, including adrenaline. The effects were also much more variable than in the cat and sometimes the rise of pressure was preceded by a transient fall. Doses of 10 mg. or more usually produced a purely depressor response. The pressor effect was more liable to diminish rapidly with successive doses in rabbits than in cats.

A typical result is illustrated in Fig. 7, where the upper record is a tracing of the respiration. A dose of 4 mg. of β -cyclohexylethylamine produced a preliminary fall of pressure from 90 to 80 mm. Hg, followed

by a gradual rise until, 4 min. after injection, the pressure was 102 mm. Hg. This rise lasted for several minutes. Respiration increased from 28 to 34 per min. but returned to the normal rate in 4 min. A comparison of this tracing with Figs. 3C and 5B, in each of which the same dose of cyclohexylethylamine was given as was administered to the rabbit in this experiment, will show the insignificant effect on the blood pressure in the rabbit as compared with the pronounced pressor effect of the same dose in a spinal cat.

Uterus

The isolated uterus of the non-pregnant cat is a convenient tissue for determining peripheral sympathomimetic action as it is relaxed by adrenaline. The actions of this series of compounds showed considerable

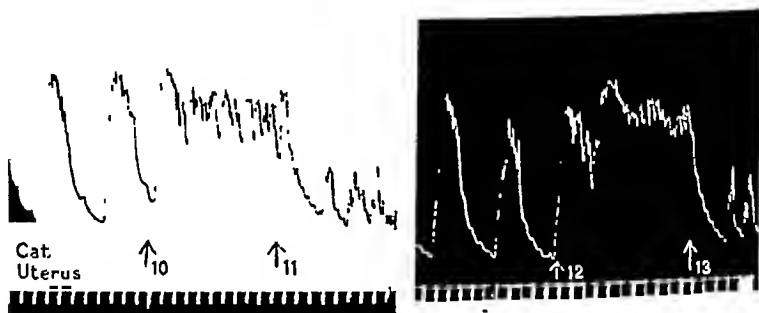


Fig. 8. Isolated non-pregnant cat's uterus. Showing stimulation by β -cyclohexylisopropylamine (1:25,000) at 10, and by cyclohexenylisopropylamine (1:10,000) at 12. Contrast the inhibitor effect of adrenaline (1:10⁷) at 11 and 13.

variations in their effect on the uterus. The most usual effect was a stimulant one, produced by concentrations of 1:50,000 to 1:10,000. This is illustrated in Fig. 8. The uterus in Locke's solution showed regular rhythmic movements. At 10 cyclohexylethylamine was added to make it into a concentration of 1:25,000. This produced a marked increase in the tone of the muscle with superimposed more frequent rhythmic movements. The addition of adrenaline at 11 (1:10⁷) produced prompt relaxation of the uterus. After being twice washed with Locke's solution, the uterus regained its previous rhythm. The addition of cyclohexenylisopropylamine at 12 caused rise of tone, which was again removed by adrenaline (1:10⁷) at 13. Relaxation of the uterus occurred in some experiments with weaker solutions, especially with the β compounds 14 and 16, and it is difficult to account for this as being due to anything other than a sympathetic stimulation. The margin between the stimulant

and inhibitory concentrations was often a sharp one, as is illustrated in Fig. 9. Here relaxation of the uterus was produced by β -phenylethylamine in concentrations of 1:100,000, 1:50,000, and 1:35,000 but 1:20,000 produced a well-marked increase in tone. The second horn of the same uterus after keeping at 0° C. overnight, responded to all concentrations from 1:250,000 to 1:5,000 by contraction. Three other fresh uteri were also stimulated by concentrations from 1:50,000 to 1:10,000. Evidently, therefore, not only do different uteri respond differently to the same concentrations of this compound but the same uterus may give a different response according to the conditions under which it is kept before testing. Somewhat similar variable results have been obtained

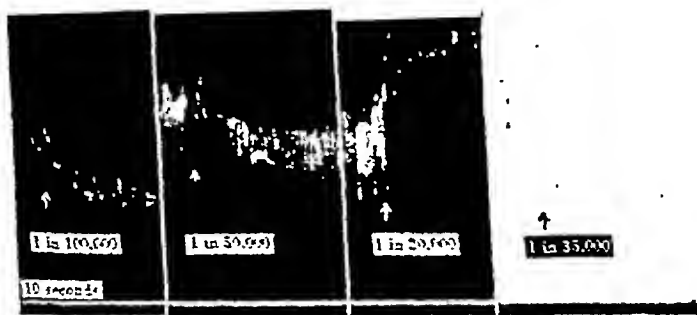


Fig. 9. Isolated non-pregnant cat's uterus. Showing contraction with β -phenylethylamine 1:20,000, but relaxation with weaker solutions.

with other compounds, in sharp contrast to the prompt and consistent inhibition of the non-pregnant cat's uterus that is produced by adrenaline.

As the rabbit's uterus is stimulated by adrenaline, the response it gives is not of much assistance in deciding between a direct stimulant action on muscle and a true sympathetic stimulation. Only a few experiments were done on the rabbit's uterus and they showed that the present group of compounds resembles others previously investigated in stimulating the contractions of this organ.

The isolated guinea-pig's uterus was powerfully stimulated by β -phenylethylamine (1:25,000) and by β -cyclohexylethylamine (1:10,000). In both cases adrenaline produced prompt relaxation.

Intestine

The isolated intestine of the cat gave responses somewhat similar to those of the non-pregnant uterus of this animal. Solutions of 1:50,000 to 1:10,000 produced increase of tone of the muscle, while weaker solu-

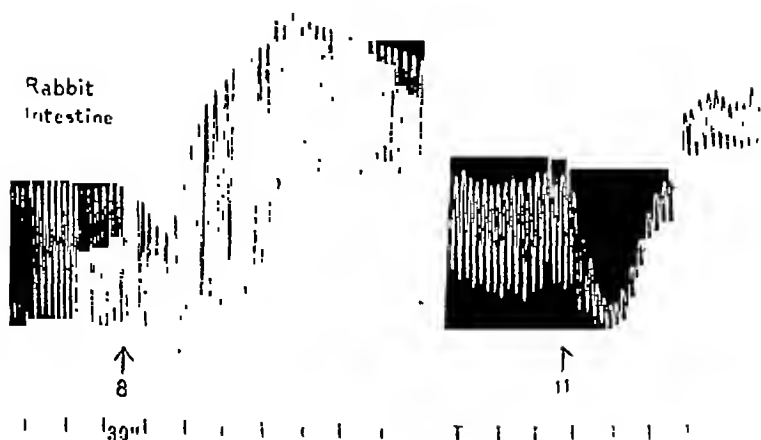


Fig. 10. Isolated rabbit's intestine. Showing transient depression followed by stimulation with both β -phenylethylamine at 8 and β -cyclohexylethylamine at 11.

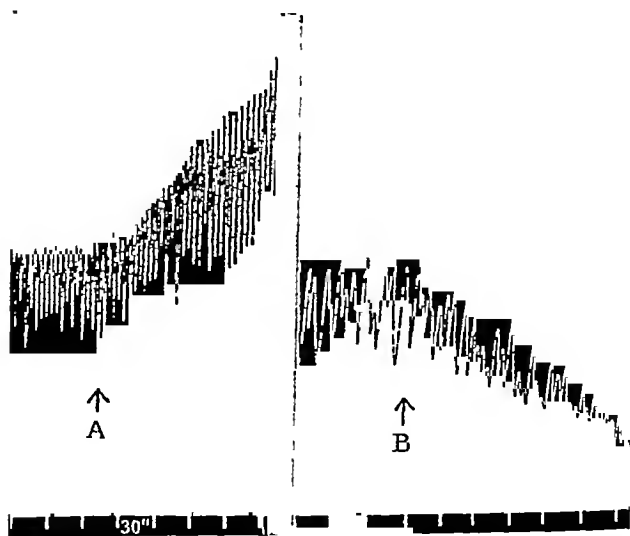


Fig. 11. Isolated intestine of rabbit (A) and of cat (B). Showing stimulant effect of β -phenylethylamine (1:200,000) on the rabbit's intestine as contrasted with the inhibitor effect of the same concentration on the cat's intestine. Only the latter effect resembles the effect of adrenaline but is produced less promptly and completely.

tions (1:200,000 to 1:100,000) sometimes produced relaxation. Even when such relaxation occurred, it was produced much more gradually by these compounds than by adrenaline. High concentrations (e.g. 1:2,000) produced paralysis of the muscle.

In the isolated rabbit's intestine, the inhibitor effect of weak concentrations was usually absent. In one experiment with β -phenylethylamine, the weakest concentration to have any effect was 1:500,000 which produced slight stimulation, and all concentrations up to 1:10,000 produced an increasingly greater effect of the same type. A solution of 1:5,000 caused preliminary stimulation, followed by paralysis of the muscle, and still stronger concentrations paralysis only.

Fig. 10 shows a typical effect of 1:10,000 of β -phenylethylamine (at 8) and of β -cyclohexylethylamine (at 11). The two substances produce almost identical effects, a transient depression followed by a more prolonged rise of tone. Fig. 11 shows the effect of 1:200,000 of β -phenylethylamine on the intestine of the rabbit (a) and the cat (b). In the former the effect is a stimulant one, in the latter a weak inhibition.

DISCUSSION

The main object of this investigation was to determine the effects of reduction of the phenyl nucleus upon the physiological action of substances related to adrenaline. The compound nearest to adrenaline is β -cyclohexylethylamine which differs from β -phenylethylamine only in reduction of the ring, and comparisons of the action of these two have been made. As examples of the effects of alteration of the side-chain upon compounds with the reduced nucleus, the methylamine and isopropylamine derivatives have also been examined. One compound with a partially reduced ring, cyclohexenylisopropylamine, has also been examined. An α compound, α -cyclohexylethylamine, has been compared with α -phenylethylamine, though these compounds were of less interest as they are further removed from the adrenaline structure.

Of these compounds only β -phenylethylamine has had any considerable previous physiological investigation. We have used it mainly for comparative purposes and consequently do not propose to review in detail the literature on this compound, which has been discussed by Raymond Hamet [1933] and more recently by Alles & Knoefel [1938]. It may suffice to mention how some of our results supplement previous work.

Barger & Dale [1910] found that β -phenylethylamine produced "in addition to the rise of blood pressure in the spinal cat, characteristic

dilatation of the pupil, relaxation of the urinary bladder, and inhibition of the tone and rhythm of the virgin cat's uterus". They state that though the action was not completely investigated, "sufficient evidence was obtained to warrant the conclusion that the action was of the sympathomimetic type". Bry [1914] described marked respiratory stimulation in cats and clonic convulsions in rabbits; she found little effect on the blood pressure in rabbits. Barbour [1916] and Alles [1932] found the minimal lethal dose by subcutaneous injection in white mice to be 0.3 g./kg., which is sufficiently close to our figure of 0.23 g./kg. by intraperitoneal injection. Our experiments confirm the stimulant action on the central nervous system and on the respiration in the intact animal; the latter effect we have seen also in rabbits anaesthetized with urethane.

In regard to blood pressure our experiments agree with those of Barger & Dale in finding a marked pressor action in spinal cats and with those of Bry in finding a feeble pressor action in rabbits. Alles & Knoefel have also drawn attention to the comparative ineffectiveness of β -phenylethylamine as a pressor agent in the rabbit as compared with the cat or dog and point out that it is "in agreement with the reactivity of the animal to nicotine in comparison with epinephrine". Tainter [1933] found ergotamine had little effect on the pressor action of phenylethylamine though the action of adrenaline was reversed. Hoyt, Patek & Thienes [1934] found that cocaine abolished, while ergotoxine decreased but did not reverse, the pressor action of β -phenylethylamine. We have found substantially the same effects. Tainter especially has regarded the difference in response following cocaine and ergotoxine given by most compounds of this group as compared with adrenaline as indicating the absence of a true sympathetic stimulation.

In regard to peripheral sympathetic action as manifested on isolated organs containing smooth muscle, β -phenylethylamine does not act qualitatively like adrenaline. As with so many other compounds of this group, it has a stimulating action on many forms of smooth muscle that are inhibited by adrenaline. So far as the cat is concerned, weak concentrations may give an inhibitor effect on the non-pregnant uterus (as was found by Barger & Dale) as well as on the intestine, but even this effect is not constant, while stronger solutions stimulate both these tissues. Barbour [1916] stated that β -phenylethylamine relaxes the isolated non-pregnant guinea-pig's uterus, but our experiments agree with those of Hoyt *et al.* that β -phenylethylamine stimulates the rabbit's intestine and the guinea-pig's uterus, both of which are inhibited by

adrenaline. These results recall the earlier experiments of Epstein, Gunn & Virden [1932] who concluded that certain methoxyphenylethylamines stimulated the sympathetic terminations in cats but not in rodents, the action in the latter animals being one of direct stimulation of the muscle. This stimulation may be partly, as Raymond-Hamet suggests, due to a nicotine-like action, but the fact that the stimulant action of β -phenylethylamine on the isolated rabbit's gut persists after large doses of nicotine, as found by Alles & Knoefel, seems to demand also a direct action on muscle.

One reason for the divergent statements in the literature regarding actions of phenylethylamine and other compounds on isolated organs is that there may be a threefold action on smooth muscle. Very strong concentrations may cause relaxation due to paralysis of the muscle, intermediate concentrations contraction. Both these effects may be found in rodents and cats, and the former action has sometimes been confused with sympathetic stimulation. Weak solutions may cause inhibition of the intestine and non-pregnant uterus of the cat and this is probably a true sympathetic stimulation, which does not occur with the intestine of the rabbit or the uterus of the guinea-pig. As is shown in Fig. 9 in the case of the virgin cat's uterus, a concentration so high as 1:35,000 may cause relaxation, while one of 1:20,000 may cause contraction, but the effect of those concentrations is not constant in different cats, in some of whom even weak concentrations cause contraction. Even when an inhibitory effect is produced on an isolated organ by β -phenylethylamine, it is produced much less promptly and completely than by adrenaline. In the intact cat, where the condition of the organs is far more normal than in the exsected organs, small doses of many of these compounds, which give a low concentration in the blood, produce the inhibitor effects on the intestine and non-pregnant uterus more promptly and regularly than occur in the exsected organ, as was shown by Epstein *et al.* with methoxyphenylethylamines. Under similar conditions in the intact rabbit, however, the same compounds give only contraction of the intestine. The absence of sympathomimetic inhibitory effects in rabbits which are exhibited in cats cannot, therefore, be ascribed to vagaries in the behaviour of exsected tissues.

The moral of this discussion is that, when compounds of this group are compared with one another or with adrenaline in regard to their actions on isolated tissues, the results may be not only valueless but positively misleading unless due cognizance is taken (a) of the effects of different concentrations on the same tissue, (b) of the varying effects of

a particular concentration in different animals of the same species, and (c) of the different effects on the same organ of different species. It is want of recognition of these complexities that is largely responsible for the divergent statements in the literature, a particular compound being described by one observer as producing contraction, by another as producing relaxation, of particular tissue.

These points have been discussed in relation to β -phenylethylamine because more ample data are available with this compound from the work of several investigators. There seems every reason to believe that the same arguments apply to the new compounds the actions of which have been described in this paper, and which can now be more briefly reviewed.

From the basic point of view, the most important compound is β -cyclohexylethylamine (hexahydrophenylethylamine) which differs from β -phenylethylamine only in reduction of the ring. Bry [1914] recorded only three experiments with it and found that it caused acceleration of respiration in the cat, slight and mainly depressor effects on the blood pressure of the rabbit and contraction of the rabbit's uterus. Waser [1927] described a rise of pressure in the anaesthetized dog with doses of 3-10 mg., contraction of the isolated uterus of the guinea-pig with a concentration of 1:5000 and of the intestine with 1:10,000. Our more extensive investigation has shown that there is no important qualitative difference in the actions of β -cyclohexylethylamine and β -phenylethylamine, the symptoms in the intact animal, the primary effects on blood pressure in different animals as well as the effects following ergotoxine and cocaine, and the actions on smooth muscle in different organs being substantially the same with both compounds. The chief quantitative differences are that the former is more toxic to white mice, but has a less powerful pressor action in cats, than the latter.

The comparison of compounds with other alkylamine side-chains, so far as they have been available, also bears out the conclusion that reduction of the ring has surprisingly little effect on the resulting compound. Thus, in regard to toxicity and general physiological actions, β -cyclohexylisopropylamine differs very little from β -phenylisopropylamine (benzedrine). In regard to stimulant action on the central nervous system, the order of activity of compounds, methylamine < ethylamine < isopropylamine, holds good for the cyclohexyl as well as the phenyl compounds. Only one compound was available for studying the effect of partial reduction of the ring, viz. β . Δ .1.2.cyclohexenylisopropylamine. With the isopropylamine side-chain, the toxicities for mice are

in the order: cyclohexenyl, 0.09; phenyl, 0.12; cyclohexyl, 0.16. The superior activity of the cyclohexenyl nucleus could hardly have been anticipated and suggests that an examination of other derivatives of this nucleus would be interesting.

In regard to α compounds, Barger & Dale noted that α -phenylethylamine had a feeble pressor activity compared with the β compound. Our experiments confirm this and show that the same is true of the corresponding cyclohexyl derivatives. In regard to toxicity, however, the α compounds are in both cases only very slightly less active than the β compounds.

SUMMARY

The actions of some cyclohexylalkylamines have been examined and compared with the actions of the corresponding phenylalkylamines. The following are the main conclusions:

1. β -Cyclohexylethylamine stimulates the central nervous system, accelerates the respiratory movements, has a pressor activity in spinal cats equal to about one-four-hundredth of that of adrenaline, and a feeble pressor activity in anaesthetized rabbits. Strong solutions paralyse, weaker solutions stimulate, the isolated intestine and uterus of the guinea-pig and rabbit; and in these tissues the inhibitory effects characteristic of adrenaline are not produced. With the lowest effective concentrations, inhibitory effects may occur in the isolated intestine and non-pregnant uterus of the cat but less certainly than with adrenaline. No important qualitative difference was found between the actions of β -cyclohexylethylamine and of β -phenylethylamine; the former was about twice as toxic for mice as the latter, but possessed only about a quarter of its pressor activity in spinal cats.

2. With ethylamine and isopropylamine side-chains, there was likewise no important qualitative difference between the corresponding cyclohexyl and phenyl compounds. With both types of ring the central stimulant properties increase in the order: methylamine < ethylamine < isopropylamine.

3. With both cyclohexyl and phenyl rings, the α -ethylamine compounds are less active than the corresponding β compounds.

4. One compound with a partially reduced ring, cyclohexenylisopropylamine, is more toxic and more stimulant than the corresponding phenyl and cyclohexyl compounds.

5. So far as the compounds investigated are concerned, partial or complete reduction of the nucleus affects the actions of the resulting

compound quantitatively but produces little, if any, qualitative change in the physiological activity.

We gratefully record our indebtedness to Messrs Imperial Chemical Industries for supplying us with the compounds used in these experiments.

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ELECTRIC INTERACTION BETWEEN TWO ADJACENT NERVE FIBRES

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HODGKIN's recent work [1937-9] has substantiated the already strong suspicion that the propagation of the nerve impulse is maintained by the stimulating effect of the action current wave. The idea of an electric mechanism of this kind had been alive since Hermann [1899], but the recent experiments have established the order of magnitude of the effect, and have shown that the normal action current is a powerful electric stimulus capable of exciting resting regions in front of the active centre of the potential wave with a considerable factor of safety. These findings raise the questions of whether, and to what extent, an action potential travelling in one or a number of axons might affect adjacent fibres.

The possibility of such an interaction between separate, active and resting, units is of interest from several aspects. (i) Normally, local currents set up in the vicinity of an active region do not, and obviously must not, excite adjacent fibres. Some mechanism apparently is present by which, not only the further propagation of the impulse in the active fibre, but also its "isolated conduction" is ensured. (ii) A *subthreshold* effect of an action potential on an adjacent fibre must be expected, however, since some part of the local current is bound to penetrate the surrounding tissue.

The object of the present paper was to investigate the electric interaction between separate axons in a simple case, using a nerve preparation which contains two fibres only, and determining quantitatively (i) any excitability changes which might take place in one fibre during the passage of an impulse in the other, and (ii) any mutual interaction between impulses travelling simultaneously in both fibres.

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METHODS

Preparation. Using the technique described by Hodgkin [1938], two adjacent fibres were isolated from the limb nerve of a crab (*Carcinus maenas*). In the proximal part of the limb nerve, inside the meropodite, two or more large non-medullated axons (diameter about 30μ) are usually found running side by side, so that a pair of fibres held together only by sparse strands of connective tissue can be isolated from the remainder without great difficulty. The nerve was dissected in sea water using a binocular microscope of weak ($10\times$) magnification, the two fibres were then separated along part of their length so that either of them could be stimulated independently. The ends of the fibres were gripped in the tips of screw-controlled forceps (ordinary ruling pens with tips slightly bent and coated with electric insulation). The preparation was then stretched across the electrodes (bare platinum hooks, in most cases 150μ thick), and the whole system gently lifted from the sea water into a layer of aerated paraffin oil by means of a Palmer rack and pinion X block.

In this situation, each fibre is electrically shunted by the other fibre, by a little interstitial tissue, and by some sea water. In certain experiments the electric conductivity of the surrounding fluid was further reduced by soaking the preparation previously in a solution in which part of the saline was replaced by isotonic cane-sugar. As a rule, the preparation gave consistent results for several hours, much longer than required for the actual experiments. Temperatures ranged from 19 to 25°C .

Stimulation. The arrangement of stimulating and recording leads is shown in Fig. 1. For quantitative threshold tests a short thyatron

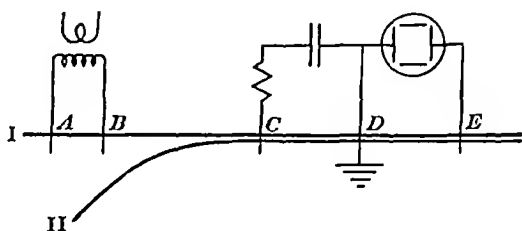


Fig. 1. Electrode arrangement for excitability measurements. I and II, fibres I and II respectively. A, B, leads for stimulation of fibre I; C, D, leads for stimulation of fibre II. D, E, recording leads connecting with amplifier and cathode ray oscillograph.

condenser discharge (time constant RC of its subsidence being $30\text{--}50\mu\text{sec.}$) was used, for ordinary stimulation a low capacitance transformer was interposed between the thyatron circuit and the nerve.

Two completely independent thyatron stimulator units utilizing thermionic trigger tripping and accelerated recharging were employed. Each of these was independently synchronized electrically with the linear sweep circuit [Schmitt, 1934] so that two stimuli of separately adjustable polarity and strength could be administered at any desired time interval, the strengths of stimuli being unaltered by variations in the time interval between the two shocks or by the frequency of sweep. The time interval between the stimuli was observed directly on the screen of the cathode ray oscilloscope; at any one setting, it was constant within the accuracy of measurement. The rate of stimulation usually employed was 1–2 per sec.

For accurate measurements of thresholds, two T pad attenuators were provided in each stimulator unit, one of logarithmic taper, the other linear and fitted with a vernier dial. The logarithmic unit permitted adjustment of the stimulus to the right order of magnitude while the linear control made accurate measurements possible.

Amplifier. The amplifier consisted of three stages of high gain (120, 120, and 500 gain/stage respectively) push-pull, cathode phase inversion, circuit [Schmitt, 1938] preceded by a head amplifier stage, again of the cathode phase inversion type, but in this case arranged as an impedance changer both with respect to input and output. This stage was mounted within a few centimetres of the nerve so that short leads could be used. The input impedance of this unit was equivalent to a capacitance of $3\text{--}4\mu\text{F}$. in parallel with approximately $100\text{ M}\Omega$ of resistance. The output impedance of the head amplifier was well below $1000\ \Omega$ so that shielded leads could be used between it and the main amplifier without producing appreciable attenuation below 50,000 c./sec.

In most of the experiments where only excitability changes were measured, this head amplifier was not used, as the frequency characteristic of the amplifier in these cases was of no consequence. With the head amplifier the overall voltage amplification was substantially constant to 5000 c./sec. and dropped about 15 % at 10,000 cycles.

RESULTS

1. *Excitability changes in a nerve fibre during the passage of an impulse in an adjacent fibre*

In the case of two adjacent axons mounted in an insulating medium the theoretical effect of an action current in one fibre on the excitability of the other can be stated in simple terms. A diagram of the local circuits, essentially identical with Hermann's well-known scheme, is illustrated in Fig. 2. In the front and the wake of the advancing active

region, the positive current lines which leave the inside of the conducting fibre (fibre I) enter the adjacent resting axon (fibre II); in the active region itself (i.e. at the points where the membrane of fibre I has become "excited" and its resting potential collapsed) the current leaves fibre II and re-enters fibre I. At all points where the membrane of fibre II is penetrated by electric currents a change of excitability must be expected, an enhancement at the "cathodal" points where the current leaves fibre II, a depression at the "anodal" points where current enters. The effect therefore will be initiated by a lowering of excitability upon which is subsequently superimposed a rise in excitability and a final depression [Katz & Schmitt, 1939].

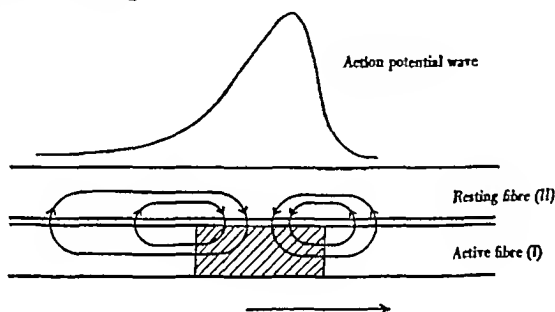


Fig. 2. Local circuit diagram, illustrating the penetration of fibre II by the action currents of fibre I. The shaded area indicates the "active region" of fibre I. Note that the direction of the penetrating current reverses twice.

A more accurate idea of the time course of these expected changes can be obtained by analysing the form of the monophasic action potential of fibre I. It is obvious from Fig. 2 that the external current which penetrates fibre II has essentially the same temporal and spatial distribution as the transverse "membrane current" in fibre I. The latter, however, is proportional to the second derivative d^2P/dx^2 (or d^2P/dt^2) of the monophasic action potential P with regard to distance x (or time t) of its propagation. (This has been shown by Cremer [1929] and others; see e.g. A. V. Hill, 1934; Cole & Curtis 1939.) The function d^2P/dt^2 can be obtained by graphical or by electric differentiation of the monophasic action potential [Schmitt, 1939a; see Fig. 5], the first derivative being obtained by placing the lead-off electrodes very close together (about 0.2 mm.), the second derivative by electrically differentiating this first derivative (Fig. 5).

The above expectations were tested in the following way. Fibre I, as illustrated in Fig. 1, was stimulated at AB ; at various moments after-

wards, while the impulse approached and passed through the region *CDE*, the threshold of fibre II was tested by a short condenser shock (*RC* 30 to 50 μ sec.) applied to *CD*, with cathode at *D*. The time interval between the stimuli was measured from the distance between the shock artefacts on the oscilloscope screen; the threshold index was the appearance of the propagated (diphasic) spike of fibre II.

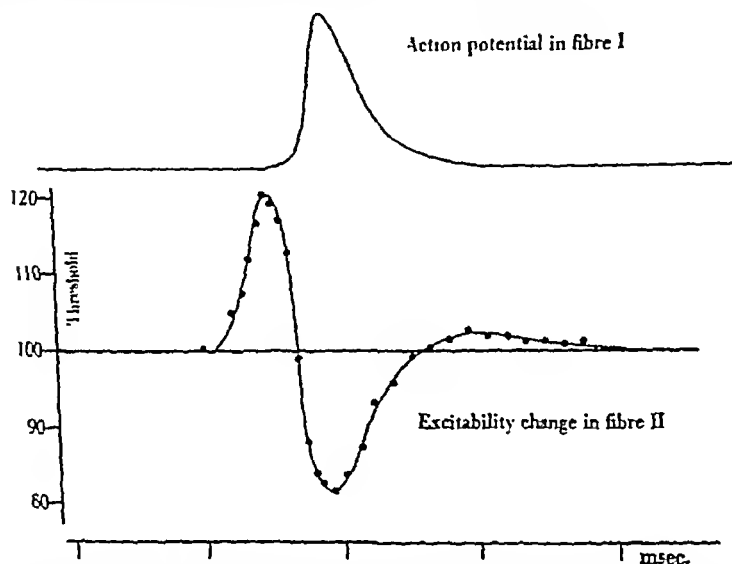


Fig. 3. Excitability changes in fibre II during the passage of an impulse in fibre I (the time course of the latter is illustrated in upper part of figure). Abscissae: time in msec. Ordinates: threshold intensity of fibre II (for a brief shock), in percentage of its "resting threshold".

TABLE I. Peak values (in percentage of "resting threshold") of the three successive phases of excitability change

	1st phase (17 exp.)	2nd phase (17 exp.)	3rd phase (12 exp.)
Mean	117 (110*-122)	89 (82-95)	102 (100-104)

* This exceptionally low value was found in a case in which fibre I was considerably smaller than fibre II.

The observed excitability change consisted of two or three successive phases: a depression at first, suddenly changing into a "supernormal" phase, which was often followed by another period of slightly reduced excitability. A typical result is shown in Fig. 3. The peak values of the three successive phases are given in Table I.

It was found sometimes that this triphasic cycle was followed by another period of slightly raised excitability. This, however, was only observed if, during a period of super-

normal recovery, fibre I became more excitable than fibre II, and so was capable of reducing the threshold of fibre II by its second action potential (see below).

Control experiments. These results, though self-consistent, might be affected by a number of errors. Excitability changes in the resting fibre might conceivably be due, for instance, to a direct effect of the stimulus transmitted by electrotonic currents or through external stray capacities, or to circulation of current through the input leads of the amplifier. Both of these possibilities were eliminated by the fact that neither a reversal of the stimulus, nor a reversal of leads *C* and *E* affected the result. In the former case, there was merely an increase of the conduction interval between the application of shock I, and the arrival of the impulse with accompanying excitability change at *D*. In the latter case, the excitability change remained unaltered, while the action potential was, of course, recorded somewhat sooner (at *CD* instead of *DE*).

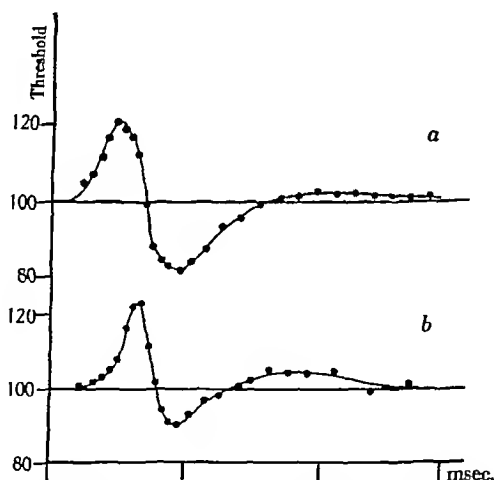


Fig. 4. Effect on the excitability curve of interchanging fibres I and II. Co-ordinates as in Fig. 3. *a*, threshold changes measured in the *more* excitable fibre; *b*, in the *less* excitable fibre. For further explanation see text.

The observed result depends to some extent, however, upon the relative "resting thresholds" of fibres I and II. The preferable arrangement is the one in which fibre II (the "test fibre") has the lower threshold; otherwise one cannot really determine the "base-line" of the excitability curve, viz. the resting excitability undisturbed by action currents in the adjacent fibre. There is strong indication (see also p. 475 above) that, at the point of stimulation, the less excitable axon receives

some encouragement from its active neighbour, and that its apparent resting threshold is really lowered, by a few per cent, by the slightly preceding action potential in the more excitable element. It is easy to appreciate that, with this latter arrangement, the phase of threshold lowering will be apparently reduced in size, since the base line from which the changes are measured is already lowered to some extent; conversely, the phases of increased threshold may be relatively exaggerated. This was verified in several experiments, as illustrated in Fig. 4. As a whole, however, the effect, while changing in details, is not altered essentially by an interchange of fibres I and II.

Time relations between action potential and excitability change. A further observation which requires some comment is the consistent divergence between the durations of the action potential and the accompanying excitability change in the neighbouring fibre. The excitability change precedes, and outlasts, the action potential by about $\frac{1}{2}$ msec.

The longer persistence of the excitability change is, of course, nothing unusual; as in ordinary electric stimulation, the "local excitatory disturbance" subsides only gradually after the stimulating current has ceased. The earlier start of the observed change also conforms to general experience [see e.g. Blair & Erlanger, 1936]. It is known that the response to a threshold shock does not propagate at full speed immediately, but requires about $\frac{1}{2}$ msec. to move away from the point of stimulation. During this interval, a relatively weak anodal shock is capable of preventing propagation. This is exactly what happens in the present case, with the single difference that the anodal shock is provided, not by an external source, but by the wave front of the approaching action potential (see Fig. 2). The strength of the shock applied to fibre II must, therefore, be raised even if the anodal currents, from the action potential of fibre I, arrive $\frac{1}{2}$ msec. later.

Time course and size of the effect. In a general way, the direction and temporal configuration of the observed excitability changes agree with the predictions of the local circuit theory. It is important, however, to inquire whether the quantities are of the right order of magnitude.

The size of the effect depends not only upon the strength of the penetrating current, but also upon the time factor of the nerve fibre (see Hill's excitation theory [1936]). One may assume, using earlier results [Katz, 1937; Hodgkin, 1938], that the time factor k of a large crab fibre is of the order of 0.25 msec., at 20° C. Knowing the approximate configuration of the penetrating current pulse (Fig. 5), it is possible to

reconstruct the approximate time course of the expected excitability change (Hill's "local potential" [1936]).

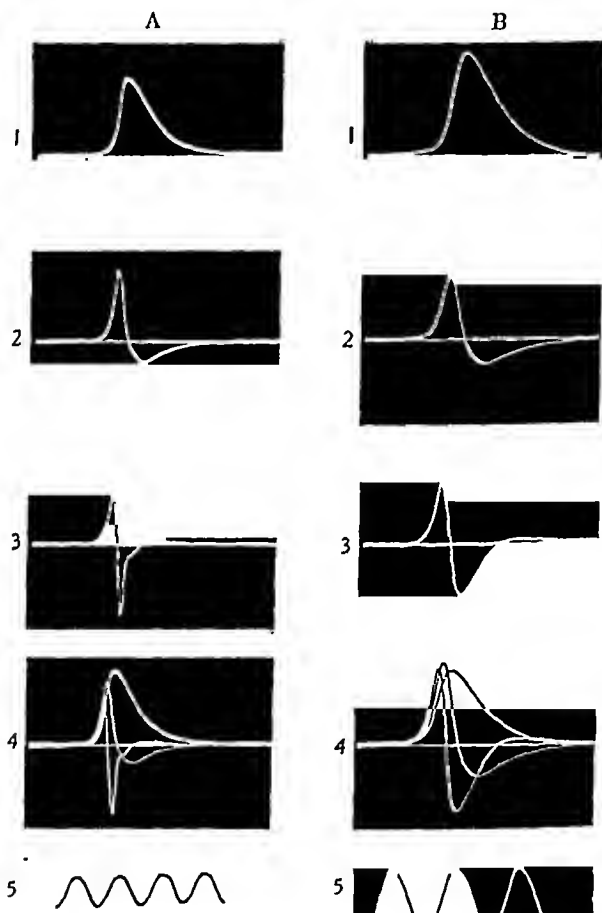


Fig. 5. Time course of "membrane current" obtained by electric differentiation (see p. 474). *A* 1, monophasic action potential, obtained by electric integration of *A* 2 [cf. Schmitt, 1939*b*]. *A* 2, diphasic action potential. Distance between lead-off electrodes 0.12 mm. *A* 3, "membrane current", by electric differentiation of (2). *A* 4, superimposed records of (1)–(3). *B*, a similar series from a different preparation. Inter-electrode distance 0.3 mm. "Imperfect differentiation" in *B* 3, giving a distorted picture of the membrane current which resembles closely the form of the excitability curve (Figs. 3, 6; see also p. 479). Time signals, 2000 per sec. Vertical scale arbitrary. Note that records *A* 3 and *B* 3 resemble curves 1 and 2 of Fig. 6, respectively.

This operation can be done graphically [Rushton, 1937; see Fig. 6], or electrically [cf. Monnier, 1934]. Record *B* 3 in Fig. 5 which was actually obtained by an imperfect electrical

differentiation of the diphasic, differential element of action potential, gives a fair picture of the excitability change, rather than of the membrane current, as this "imperfect" differentiation is substantially the process employed by Monnier and Rushton.

Let us consider two limiting cases: (i) The time factor k is large compared with the duration of the action potential. In this case, the "local potential" is obtained by integrating the membrane current, i.e. its time course is represented by the diphasic action potential, as recorded in Fig. 5. (ii) The time factor is very small; in this case, the "local potential" would follow the tri-phasic changes of the membrane current without appreciable distortion. In practice, some intermediate result must be expected (Figs. 3, 4, 6).

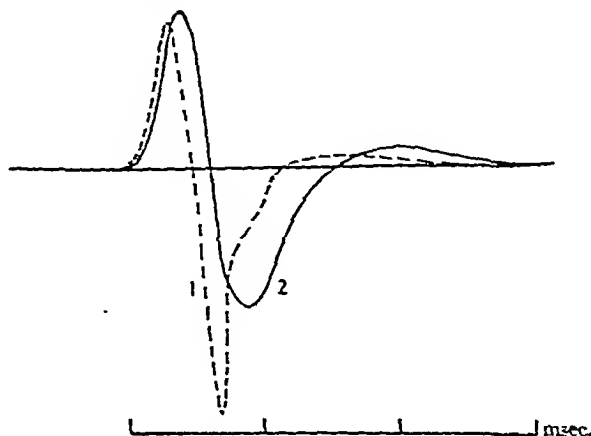


Fig. 6. Excitability change, and stimulating current obtained by graphical analysis. Curve 2 (full line): observed threshold changes. Curve 1 (broken line): time course of stimulating current which, according to Hill's [1936] theory, would produce the observed excitability changes. A time factor k [Hill, 1936] of 0.25 msec. has been assumed. Curve 1 was derived using Rushton's [1937] graphical method. Note the resemblance between curves 1 and 2, and records A3 and B3 of Fig. 5, respectively.

The observed excitability curve is very similar to the one reconstructed from the membrane current (Figs. 5, 6); it should be noted, in particular, that the amplitude of the first depression phase is, if anything, greater than that of the period of raised excitability (cf. also Table I), in contrast to the undistorted "membrane current" picture.

To obtain an estimate of the absolute magnitude of the changes to be expected, we must make a number of assumptions regarding the external distribution of the local action currents. From recent measurements by Hodgkin & Rushton (personal communication) it appears that the conductivities, for longitudinal currents, of the "inside" and "outside" of an isolated crustacean axon are about equal. In the case of two fibres, one might expect, therefore, that at least two-thirds of the external action current is taken up by the interstitial fluid and connective tissue while the

remaining third might penetrate the resting fibre. Actually the penetrating fraction would be further reduced by the considerable transverse impedance of the resting membrane [see Cole & Hodgkin, 1939].

Another even more important factor which limits the excitatory effect of the external action currents is the immediate reversal of the membrane current, as soon as it has reached threshold strength in the active fibre [cf. Cole & Curtis, 1939]. This automatic "cut-off" mechanism provides an effective protection of the surrounding elements against any very drastic disturbance.

Taking both factors together, we may assume that rather less than one-third of threshold current enters the resting fibre; assuming further that the excitabilities of both fibres are about equal, this should produce an initial threshold rise of rather less than one-third. The observed values (up to 20 %, see Table I) do not differ very much from this estimate. The subsequent phase of threshold lowering might be of this same order of magnitude but cannot be much larger, as has been pointed out. We can understand, therefore, that even under the present conditions which appear to be ideally suited for electric interaction of fibres, the external stimulating effect of the impulse stays far below threshold, and "isolated conduction" is safeguarded by a good margin.

There seemed some possibility that, by reducing the size of the platinum electrode *D* (Fig. 1) (to 40μ instead of 150μ), and by carefully avoiding any accumulation of sea water at the point of contact, the observed effect might become larger, due to removal of the shunt constituted by the width of the electrode and its associated droplet of liquid. This, however, was not the case, presumably because the test shock, in any case, excites at the "inner" edge of the applied metallic, or fluid, contact only. In another attempt to obtain an increased effect, three fibres were used, and the excitability change in one axon measured, while an impulse was travelling in both adjacent fibres. This, again, did not change the result; one could, however, hardly expect it to do so, unless the two action potentials happened to be exactly synchronized.

One should, however, expect a distinct change if the resistance of the outside fluid were raised artificially, and consequently a larger fraction of the action current were forced to penetrate the shunting fibre. This can be verified by soaking the preparation in a mixture of sea water and isotonic cane-sugar before lifting it into oil. In this way an effect was obtained. Replacing half the saline by isotonic sucrose increased the size of the threshold change reversibly, from 22 to 30 %. The effect will be dealt with in more detail in § II below.

II. *Mutual interaction of impulses in adjacent axons*

The previous observations show that, with each pulse of activity travelling in fibre I, an accompanying wave of excitability changes sweeps along the adjacent fibre II. If impulses exist simultaneously in both fibres, one may expect a slowing or speeding to take place depending, at any particular moment, upon the relative phase of the two impulses.

(i) If impulse II lags a little behind impulse I, so that the wave front of II coincides with the active phase of I, then clearly the local membrane currents which leave fibre II are reinforced by the external currents which re-enter fibre I, and the propagation of impulse II will be facilitated. In other words, the stimulating process in fibre II takes place during a period of raised excitability, induced by the action currents of fibre I.

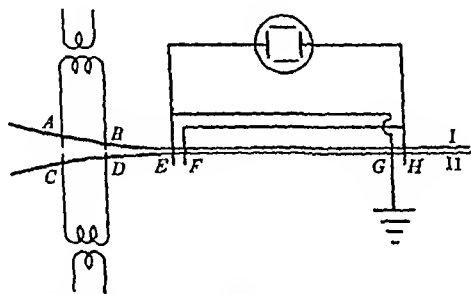


Fig. 7. Electrode arrangement for the measurement of velocity changes. I and II, fibres I and II respectively. A, B and C, D, stimulating leads; E, F and G, H, two pairs of recording leads. The action potentials of each fibre are recorded before and after the impulses have been travelling beside each other.

(ii) If, however, impulses I and II happen to be accurately in step, then one must expect a mutual interference of the wave-front currents. Stimulation in each fibre would then occur while its excitability is being lowered by the influence of its neighbour and, therefore, propagation would be slowed. One can express it also in the following terms: the action currents of neither fibre can penetrate the adjacent axon because the fibres are mutually equipotential. The only return path available, therefore, is the interstitial tissue; in other words, the synchronous activity of neighbouring fibres acts like an increase in external impedance.

These expectations are borne out by experiment. Two axons were dissected, as before, but mounted in a different way, illustrated in Fig. 7. Fibre I was stimulated through electrodes AB, fibre II through CD, and

the action potentials were led off from two pairs of electrodes, connected together as shown in Fig. 7. In this way, as pointed out by Schmitt [1939a], one can obtain, from a single record, a very accurate measurement of the conduction velocity. In each fibre, a diphasic wave is recorded when the action potential passes the region *EF* and again when passing *GH*. The velocity of the impulse is obtained from the time interval between the two diphasic waves.

The distance *FG* was 12–15 mm. The action potential occupied, at any moment, only about 5 mm. of nerve and required about 3 msec. to travel from *F* to *G*. During this interval, any excitatory disturbance which might have been caused in region *GH* by being connected to leads *EF* would have disappeared. It was considered unnecessary, therefore, to insert any extra resistances in the lead-off circuit.

The interval between the shocks to fibres I and II was adjusted so that the action potentials could be given any desired phase relationship when arriving at the "junction" *E*. With each interval, three different records were superimposed, to facilitate the comparison (see Fig. 8): (i) and (ii), the action potentials of fibres I and II, separately excited, and (iii), the combined potential wave due to simultaneous stimulation.

The results are illustrated in Figs. 8 and 9. It is obvious that the combined action potential recorded at the first pair of leads (*EF*) is simply the sum of the two separate waves without any change in form or position. This was true only if the fibres were kept apart between the stimulating and first pair of recording leads. If the fibres "joined" a few mm. before the leads *EF*, the combined potential wave differed from the sum of the individual waves and showed that an interaction had taken place before the impulses reached the first pair of recording leads.

The second potential pulse, recorded from the points *GH*, reveals the sign and extent of interaction which has occurred while the impulses were propagating beside each other through the intermediate stretch *FG*. The size and direction of this mutual effect depended upon three principal factors: (i) the difference in velocities of impulses I and II, (ii) the phase at the start (i.e. at *EF*), and (iii) the electric conductivity of the interstitial fluid.

The first factor varied from experiment to experiment in an uncontrollable fashion, depending in part upon the relative diameters of the two axons [Erlanger & Gasser, 1937]; the other factors could be varied at will.

Obviously, if the velocities of impulses I and II are widely different, their mutual interaction cannot be very extensive, since the faster action potential will stay side by side with the slower one only for a very short

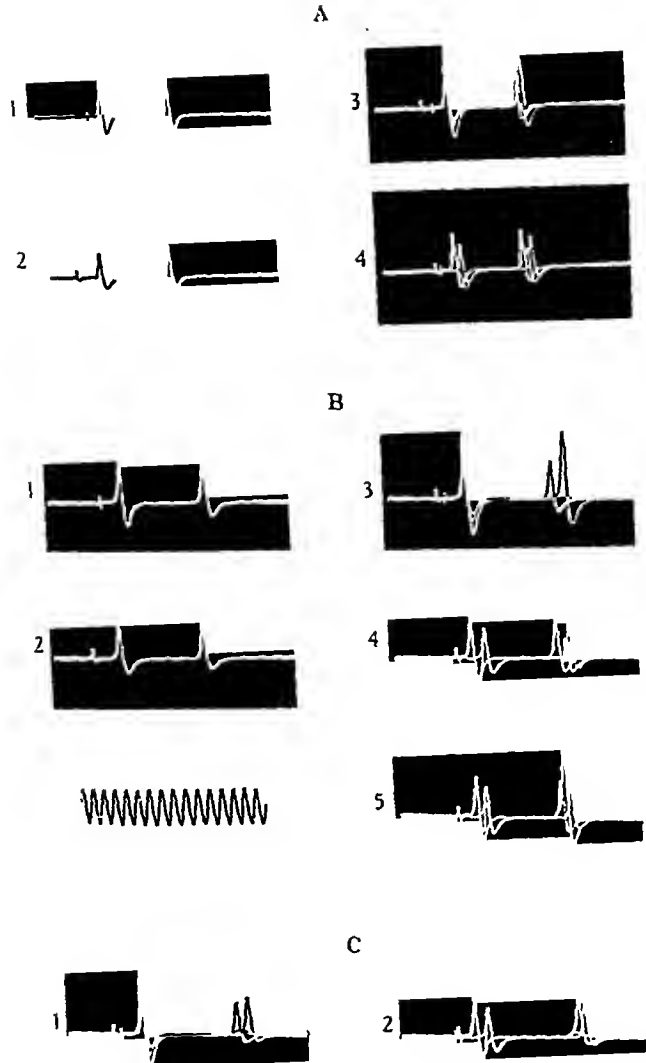
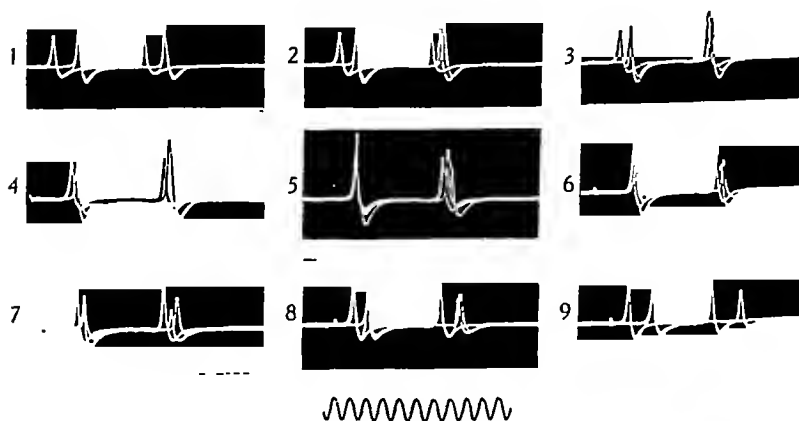


Fig. 8. Mutual interaction of impulses in two separate fibres (see text). *A*, normal preparation. *A* 1, action potential of fibre I, recorded first as it passes electrodes *E*, *F*, then as it passes *G*, *H* (see Fig. 7). The interval between the two spikes is 2.72 msec., the intermediate length of nerve 13.5 mm., the conduction velocity, therefore, 4.95 m./sec. *A* 2, action potential of fibre II. *A* 3 and 4, superimposed records of individual, and combined, action potentials, travelling together in different phase relationships. *A* 3 shows the delay of the combined wave if the impulses are "in step"; *A* 4, the speeding of the later impulse. *B*, Same preparation, in "50 isotonic sucrose/50 sea water". The interaction is more marked; individual conduction is slowed. *C*, same as *B*, but the electrodes *G*, *H* have been moved just beyond a region in which one of the impulses became blocked (only a small monophasic remainder being recorded at *GH*). The combined potential wave at *GH* can be recognized by its larger size. Mutual slowing in *C* 1, speeding of the later impulse in *C* 2. Time signal 2000 per sec.

time and will soon leave it behind. The smaller the velocity difference, the longer will the impulses stay together and, therefore, the better the chances of a thorough interaction.

A



B

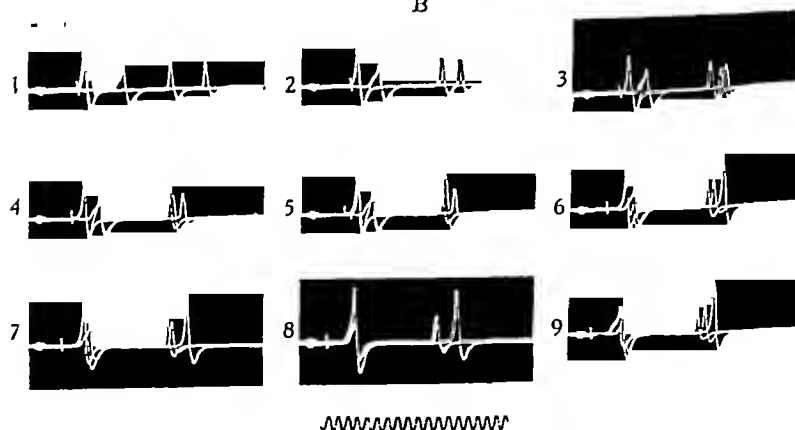


Fig. 9. Interaction of two nerve impulses (see text). *A*, in normal nerve; *B*, with "50 sucrose/50 sea water". Time signals 2000 per sec. *A* 1-9, the phase relationship of the two impulses at the start is varied progressively. Mutual slowing in 4 and 5, speeding of later impulse in 2 and 7. *B* 1-9, " $\frac{1}{2}$ sugar" preparation. The effects (especially *B* 8 and *B* 4) are more marked.

In a case, for instance (Fig. 8), in which the velocities of the individual impulses differed by 5 %, the following changes were observed: the quicker impulse, if it was initially behind the slower one, gradually

caught up with the latter. When it had approached it within about $\frac{1}{2}$ msec., it became accelerated until the two impulses had come nearly in step, it then slowed down to less than its initial speed, which it recovered as it proceeded to overtake the slower impulse. However, at this moment, the slower impulse started to speed up a little and in a certain, rather critical, position the velocities of both impulses became equal. From now onwards, the two impulses travelled together at the same speed, and remained in this peculiar, slightly "out-of-phase", position. The quicker impulse acted rather like a pacemaker, dragging along its slower neighbour. As a whole, therefore, if the velocities are only slightly different, the electrical interaction of the two local circuits suffices to make them very nearly, or exactly, equal and to bring about a more or less persistent "synchronization" of the two impulses, though in a characteristic "out-of-step" position.

If the velocities differ by more than about 10 %, the same process of speeding and slowing takes place, but is not sufficient normally to equalize the speeds; sooner or later, therefore, the faster impulse will completely escape from the slower one.

If the individual velocities are equal, the interaction tends to pull the impulses entirely "in step", even if they started somewhat out of phase. The impulse which lags behind is accelerated, catches up, and then a mutual slowing occurs which is most marked in the final, perfectly synchronized, position.

If the two impulses are travelling perfectly "in step", and if the electric conductivities and action potential patterns of both fibres are identical, then the action currents cannot cross from one fibre into the other, but are restricted to the interstitial fluid. This is equivalent to an increase of the resistance on the outside of the nerve fibre which leads to a slowing of the impulse. This effect in single fibres has previously been

TABLE II. Effect of reduced salinity on interaction

Sea water/isotonic cane-sugar	Individual velocity of quicker fibre in m./sec.	Maximum slowing observed during simultaneous conduction in both fibres, %
100/0	5.35	7.7
50/50	3.45	27
33/67	3.05	39
25/75	2.5	45

shown by Hodgkin [1939]. A larger change may be expected if the resistance of the interstitial fluid is raised by the substitution of isotonic cane-sugar solution for part of the sea water. In this case, the adjacent

fibre becomes a more important shunt for the action currents of each axon, and consequently the mutual slowing should be increased. This was verified experimentally (Figs. 8, 9, Table II). The lower the electric conductivity of the interstitial fluid, the more marked was the interaction, in particular the mutual slowing, of two synchronized impulses.

Finally two phenomena may be described which, though observed casually, and under abnormal conditions, are a corollary of the previous results. It was found that an impulse in one fibre was able to help its neighbour across a blocked region if the impulses were set up together and the block was not too strong. Conversely, a fibre which exhibited a hyperexcitable region was started off by the action current in the adjacent axon if it arrived during a supernormal recovery phase.

DISCUSSION

The results described are entirely consistent with the "local circuit theory" of nervous conduction as proposed by Hermann and Cremer, and recently confirmed by Hodgkin and others. The interpretation of the phenomena, in terms of local action currents, entering and leaving adjacent fibres, has been satisfactory, and the good agreement between the predicted and observed effects may be regarded as a further confirmation of Hermann's theory.

The question of stimulating effects of the action currents, and of interaction between separate conducting elements, is almost as old as electro-physiology (one need only refer to the "rheoscopic frog", and to Hering's experiments on the "secondary excitation" of one nerve by another [see Biedermann, 1895]). More recently, Blair & Erlanger [1932] reported that the action potential wave in one part of the dog's phrenic nerve does not appreciably alter the excitability of the other part, while Jasper & Monnier [1938] found that a non-medullated crab nerve, which is on the verge of spontaneous activity, may be stimulated by impulses running in an attached crab nerve. Quantitative observations, however, were not available. The present experiments show that, even under most suitable electrical conditions, the effects of an impulse in one fibre on another non-medullated nerve fibre remain well below threshold (pp. 479-480). To what extent these observations are applicable to the conduction of impulses *in situ*, and to medullated nerve, cannot be said at present. The results described may have some bearing on the phenomena of "synchronization" and "simultaneous firing" of adjacent nerve or muscle fibres, which have not infrequently been described; but it should be remembered that such effects are usually associated with abnormal alterations (e.g.

local hyperexcitability, influence of a nearby injured region; see Adrian [1930], Blair & Erlanger [1932]), which make them less comparable with the present case. The "rheoscopic frog" and Hering's experiments show that under certain conditions a medullated nerve can be excited by the action currents of a muscle or another medullated nerve, but it is equally certain that normally nothing of this kind occurs. Without a sufficient knowledge, in each particular case, of the arrangement of the local circuits, of the distribution of the action currents, and of the excitability of the tissue, it is impossible to make well-founded predictions about either sign or magnitude of the supposed effects. This applies also to the electric theories of synaptic transmission which have been put forward from time to time without, however, much attention having been paid to these factors.

SUMMARY

A preparation consisting of two adjacent fibres was isolated from the limb nerve of the crab *Carcinus maenas*.

During the passage of an impulse in one fibre, subthreshold excitability changes take place in the adjacent fibre. These were measured by applying brief test shocks.

As the potential wave in the active axon approaches, the excitability of the resting fibre is at first reduced, then quickly increased above normal, and finally passes through a second slower period of slight depression. These changes are explained as being due to a penetration of the resting fibre by external action currents from the active fibre, remembering that the direction of these currents, with respect to the nerve membrane, reverses twice and that current lines which leave the active fibre enter the adjacent resting fibre.

When impulses are set up simultaneously in both fibres, a mutual interaction takes place producing various combinations of speeding and slowing, depending upon the phase relationship between the two impulses.

If the impulses are advancing entirely "in step", their local action currents interfere with each other, and the propagation velocity is reduced. If one impulse slightly precedes, it accelerates the conduction rate of the lagging action potential. This effect leads to a "synchronization", and to equalization of speeds of the impulses, if their individual velocities differ only slightly.

The alterations of conduction rate are consistent with the changes of excitability induced by each action potential in the neighbouring fibre, and can be explained by simple summation of the local action currents contributed by the two fibres.

The observed changes can be increased by soaking the axons in isotonic solutions of reduced salinity (sea water/cane-sugar mixtures). In this way, the conductivity of the interstitial fluid is reduced and a larger fraction of the action currents diverted into the adjacent fibre.

The quantities of the observed effects are of an order of magnitude consistent with the electric theory of nervous conduction.

In spite of the powerful stimulating effect of the action potential wave in the conducting fibre itself, the external effects are small, and a large safety margin is provided for the "isolated conduction" of the impulse in each individual fibre. The principal factors by which this mechanism is ensured are discussed.

We are greatly indebted to Prof. A. V. Hill for his kind hospitality and encouragement and to Mr J. L. Parkinson for his invaluable assistance.

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THE LIPOTROPIC ACTION OF METHIONINE

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In 1937 Tucker & Eckstein demonstrated that methionine exerts a lipotropic effect. This has been confirmed in our laboratory [Best & Ridout, 1938] and by Channon, Manifold & Platt [1938]. The latter workers have shown that methionine, under the conditions of their experiments, exerted very little effect upon the deposition of fat in the liver unless the basal diet was such that large amounts of fat were deposited in the livers of the control animals. We have been interested in the lipotropic effects of *d*- and *l*-methionine and in the failure of large doses of the racemic mixture to produce greater effects than small doses under certain experimental conditions.

METHODS

White rats of the Wistar strain, av. wt. 200 g., were used. The liver fat was estimated by direct saponification and the results are expressed as total fatty acids plus unsaponifiable matter per 100 g. fresh tissue. Animals of the same sex were used throughout an experiment and the figures given in the tables are the average values from groups of fifteen animals. The constituents of the basal diet are listed in Table V. The diets were all supplemented by adequate amounts of crystalline vitamin B₁ and vitamins A and D in the form of a cod-liver oil concentrate. The substance to be tested was added to the basal diet and a corresponding decrease in the amount of sucrose was made. The experimental period was 21 days. Daily records of the food consumption were made and from these the actual intake of the supplement was calculated.

RESULTS

The results of the first experiment are summarized in Table I. They demonstrate that under these experimental conditions the lipotropic

TABLE I

Diet	Av. daily food intake g.	Av. daily intake of supplement	Av. change in wt. %	Liver fat	
				g. per 100 g. rat (calc.)	%
Basal	9.2	—	-10	0.58	15.2
+0.125% <i>dl</i> -methionine	9.5	11.9 mg.	-6	0.47	11.6
+0.25% "	9.7	24.2 "	-5	0.42	10.0
+0.50% "	9.4	47.0 "	-4	0.37	9.4
+1.00% "	9.7	97.0 "	-9	0.44	10.5
+8.0% casein	9.5	0.76 g.	+3	0.62	14.2
+16.0% "	9.6	1.54 "	+9	0.42	9.6
+32.0% "	9.0	2.88 "	+3	0.25	6.2

effect of a diet containing 1% *dl*-methionine is not significantly greater than that of one with 0.125%.

Dietary casein which contains approximately 3.2% of methionine [Baernstein, 1932] has previously been shown to exert a definite lipotropic action. In this experiment 47 mg. of methionine daily exerted about the same effect as the diet containing 16% casein which provided approximately 49 mg. of this amino-acid. On the other hand, at lower or higher levels of intake of the two substances, the effects were by no means identical.

As mentioned above, Channon and his collaborators showed that the lipotropic effect of methionine was much greater when the basal diet produced a high level of liver fat in the control animals. The results in Table II show that the absence of additional effects with larger doses of methionine

TABLE II

Diet	Av. daily food intake g.	Av. daily intake of supplement	Av. change in wt. %	Liver fat	
				g. per 100 g. rat (calc.)	%
Basal	9.2	—	-6	0.89	20.5
+0.50% <i>dl</i> -methionine	9.3	46.5 mg.	-1	0.55	13.3
+1.00% "	9.4	94.0 "	-6	0.64	15.5
+2.00% "	9.5	190.0 "	-11	0.68	16.5
+16% casein	9.0	1.44 g.	+2	0.30	8.5
+32% "	8.6	2.76 "	+4	0.23	7.0
+0.08% choline	9.9	7.9 mg.	-6	0.30	8.3
+0.16% "	9.3	14.9 "	-5	0.24	6.6

can still be demonstrated when the control value of liver fat is high. The diet containing 2% *dl*-methionine did not lower the liver fat as much as the one with 0.5%. In this experiment the diet containing 16% casein caused a greater decrease in liver fat than could be attributed to its methionine content. The choline equivalent of the dietary casein was about 5 mg. per g. casein. This value is the same as that previously determined.

TABLE III

Diet	Av. daily food intake g.	Av. daily intake of supplement mg.	Av. change in wt. %	Liver fat	
				g. per 100 g. rat (calc.)	%
Basal	8.0	—	- 11	1.06	25.7
+0.06% <i>DL</i> -methionine	8.7	5.2	- 5	0.82	18.0
+0.50% " "	8.3	41.5	- 3	0.57	13.4
+0.06% <i>D</i> -methionine	9.2	5.5	0	0.94	19.7
+0.50% " "	8.6	43.0	- 4	0.44	10.9
+0.06% <i>L</i> -methionine	8.9	5.3	- 4	0.69	16.0
+0.03% choline	8.3	6.6	- 6	0.21	5.9
+0.02% " "	8.1	1.6	- 10	0.58	16.3
+0.01% " "	8.9	0.9	- 14	0.71	17.7
Basal	9.5	—	- 10	0.77	18.3
+0.06% <i>DL</i> -methionine	9.3	5.6	- 8	0.56	14.3
+0.06% <i>D</i> -methionine	8.5	5.1	- 8	0.60	14.3
+0.06% <i>L</i> -methionine	9.5	5.7	- 8	0.57	14.3
Basal	8.8	—	- 6	0.73	18.6
+0.15% <i>DL</i> -methionine	9.3	13.9	- 4	0.73	16.7
+0.25% " "	9.1	22.7	- 4	0.63	16.0
+0.15% <i>D</i> -methionine	8.8	13.2	- 4	0.60	15.6
+0.25% " "	8.7	21.7	- 5	0.49	13.7
+0.15% <i>L</i> -methionine	8.7	13.0	- 5	0.57	14.7
+0.25% " "	9.1	22.7	- 5	0.57	14.9

In the third experiment (Table III) the effects of *D*- and *L*-methionine are compared with that of the racemic mixture and with various doses of choline. The results indicate that both *D*- and *L*-methionine are active and that their effects are essentially similar to that of the mixture.

TABLE IV

Diet	Av. daily food intake g.	Av. daily intake of supplement	Av. change in wt. %	Liver fat	
				g. per 100 g. rat (calc.)	%
Basal	9.5	—	- 6	0.73	18.6
(1) +30% casein	8.5	2.55 g.	+3	0.25	7.1
(2) +0.15% choline	8.7	13.0 mg.	- 5	0.24	7.8
(3) +0.10% cystine	8.4	84.0 "	- 5	0.71	16.8
+0.96% <i>DL</i> -methionine		80.7 "			

In the last experiment (Table IV) cystine, which has been shown to increase liver fat [Curtis & Newburgh, 1927; Beeston & Channon, 1936], and methionine were both added to the basal diet in amounts equivalent to those provided by a diet containing 30% casein [Tucker & Eckstein, 1937]. The effect of this diet was then compared with one containing 30% casein and also with one in which choline was added to the basal diet in an amount equal to the choline equivalent of the casein. The results in Table IV show that the mixture of cystine and methionine exerts an

insignificant effect in comparison with the casein diet which provided the same amounts of these amino-acids. The composition of the basal diet and of the others referred to in Table IV is described in Table V.

TABLE V. Composition of diets listed in Table IV

	Basal %	(1) %	(2) %	(3) %
Meat powder*	5	5	5	5
Casein†	—	30	—	—
Cystine	—	—	—	0.10
<i>dl</i> -Methionine	—	—	—	0.98
Beef dripping	40	40	40	40
Sucrose	48	18	47.85	46.94
Agar	2	2	2	2
Salt mixture‡	5	5	5	5
Choline	—	—	0.15	—
Cod-liver oil concentrate	+	+	+	+
Vitamin B ₁	+	+	+	+

* For preparation, see MacLean, D. L., Ridout, J. H. & Best, C. H. [1937].

† Fat-free and vitamin-free, obtained from British Drug Houses.

‡ See McCollum, E. V. & Simmonds, N. J. [1918].

DISCUSSION

Our results confirm and extend those reported by Tucker & Eckstein [1937] and by Channon *et al.* [1938]. There does not appear to be a quantitative relationship between the amount of methionine ingested and the deposition of fat in the liver. The lipotropic activity of the diet containing 2% methionine was no greater than the one with 0.5%. However, if the amount of methionine is reduced to 0.06%, as in the experiments recorded in Table III, the falling off in lipotropic effect is very definite.

Channon, Loach, Loizides, Manifold & Soliman [1938] demonstrated that the lipotropic activity of protein supplements varied with the nature and amount of the basal protein. In the experiments reported in this paper, meat powder was used as the basal protein. It is possible that an increase in the dose of methionine might produce a further decrease in liver fat if a different protein were used in the basal diet.

Tucker & Eckstein [1937] at one time suggested that the lipotropic effect of a diet containing 30% casein as the only source of dietary protein was due to the opposing influences of its cystine and methionine contents. The results of their more recent experiments and of those we are now reporting do not support this interpretation. If 30% casein is added to a diet containing 5% meat powder as the basal protein, a definite decrease in liver fat occurs. If cystine and methionine are added to the basal diet in amounts corresponding to those supplied by the diet

containing 30% casein, there is little or no decrease in liver fat. Furthermore, the amount of liver fat is decreased when the amount of dietary casein is increased, but when additional quantities of methionine are added to the basal diet the lipotropic activity does not increase beyond a certain limit. This limit is reached under our experimental conditions when there are still large amounts of fat in the liver. If the lipotropic action of certain proteins is due to their constituent amino-acids, the results of these experiments suggest that other amino-acids are involved in the lipotropic activity of dietary casein. Tucker & Eckstein [1938] did not obtain an increase in liver fat when cystine was added to a diet which contained 5% gliadin as the basal protein, and they suggest that other amino-acids may exert the same effects as cystine or methionine. Our findings are therefore in agreement with those of Channon *et al.* [1938] and of Tucker & Eckstein [1938] who have emphasized the probability that other factors in addition to cystine and methionine may be involved in the explanation of the lipotropic effect of protein. Some light may be thrown on the problem by the further developments of the investigations of Du Vigneaud, Chandler, Moyer & Keppel [1939] and of Channon *et al.* [1938]. The effect of homocystine and related substances on fat metabolism is being studied by both these groups.

SUMMARY

1. Methionine exerts a definite lipotropic action (confirming Tucker & Eckstein).
2. The activities of *d*-methionine, *l*-methionine and the racemic mixture are of the same order under our experimental conditions.
3. In experiments in which relatively small doses of methionine have produced a significant fall in liver fat, increase in the dose has not caused a further decrease in spite of the fact that large amounts of fat were still present in the liver.
4. Our results support the conclusion of other workers in this field that factors other than cystine and methionine are involved in the explanation of the lipotropic effect of dietary protein.

We are indebted to Prof. W. E. Rose and to Dr Madelyn Womack for the preparation of *d*- and *l*-methionine.

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THE ACTION OF EMBRYONIC EXTRACTS ON THE SEX ORGANS

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THE experiments described in this paper were undertaken in order to find whether embryonic extracts influence the development and activity of the gonads, and particularly whether injections of such extracts into animals can lead to sterility. The experiments were started by an incidental observation made on a bull during the treatment of papillomatosis. The bull, which had received several injections of cattle embryonic extracts, lost its sexual activity. It appeared possible that this condition resulted from the treatment and that embryonic extracts had an inhibitory action on the sexual organs. A preliminary note on our results has already been published [Zironi & Meyerhof, 1937].

Embryonic extracts are known to increase the metabolism [Pugliese, 1927], growth, regeneration of tissue [Carnot, 1923] and proliferation of cells in tissue cultures [Carrel, 1913], but there are only a few observations on the effects of such extracts on the sex organs. Rabbit embryonic extracts inoculated into female rabbits produce swelling of the mammary glands brought about by multiplication of the acini and ducts, and may cause secretion of milk [Lane-Claypon & Starling, 1906]. The regression of the corpus luteum gravidarum post partum was found to be inhibited by embryonic extracts and abortions were produced in pregnant mice.

METHOD

The experiments were carried out on ninety-five guinea-pigs and ninety-three white rats and extended over a period of 2 years. We have examined in both species the effects of subcutaneous injections of embryonic extracts on the growth of the gonads, and in white rats the effects also on fertility and on the content of R. factor (Duran-Reynal's spreading factor) in the testis. The majority of the experiments were

done during the summer months in which the sexual development in the animals is most pronounced. The males and females were kept separately, if not otherwise stated. In those experiments in which the growth of the animals was examined, not only had the control group of animals the same average body weight, but care was taken that each treated animal was represented in the control group by an animal of the same body weight. The weight of the guinea-pigs at the beginning of the experiment was always under 150 g. in order to be certain that full sexual development had not been reached. In order to study the effects of embryonic extracts on sexual activity in rats, sexually fully developed animals were used. They were divided into two groups, one being used as control. Each group was divided into families consisting of one male and two to four females and the fertility of each family was controlled for a period of 2 months before the actual experiment was started. Only those families were used which produced per female and per month an average of about three young, the number of births in each family being divided by the number of females. In some series the males, in others the females only, were treated with embryonic extracts.

The extracts were usually from cattle and guinea-pig embryos. In a few experiments extracts from rat embryos were used. The cattle embryonic extract was the commercial extract "Embrionina-ISM" which is prepared in our institute. The extracts from guinea-pig and rat embryos were prepared in the following way. Animals in the second half of pregnancy were killed under ether anaesthesia, the uteri were opened through the abdomen and the embryos removed aseptically. They were ground up in a mortar under sterile conditions with sand and later with saline, 7-10 c.c. of saline being added to each gram of tissue. The extracts were kept at 4° C. for several days and then centrifuged. The supernatant fluid was filtered through Chamberland candles, and stored under ether at 4° C. or ampouled. The sterility of each extract was tested on agar and broth media before use. Micro-Kjeldahl estimations gave 127.5 mg. N in 100 c.c. of "Embrionina-ISM" and 36-56 mg. in 100 c.c. of guinea-pig embryonic extract. The injections of extract were made subcutaneously and given daily or three times a week. In those experiments in which the growth of the testes was studied the amount of cattle or guinea-pig extract used for a single injection contained about 0.26 mg. of nitrogen. In other experiments the amounts of extract given per injection varied between 0.1 and 1.5 c.c. In several series, control injections of horse and cattle serum were given in amounts containing the same nitrogen content as the dose of embryonic extract injected.

R. factor content in the testis. In order to determine the content of R. factor in the testis, fresh 10% extracts were made from testes of horses, bulls or rats. Some of the rats had undergone a treatment of injections every second day with cattle embryonic extract for a period of 5 weeks or 10 months respectively. At each injection 0.75 c.c. of embryonic extract containing 0.96 mg. of nitrogen were given. A piece from the interior of the testis was ground up with sand and distilled water, or sand and saline, and then centrifuged. The supernatant fluid was injected intradermally together with Indian ink into albino rabbits. Only fresh extracts made on the same day were used. The rabbits were shaved on the back and abdomen 8 hr. before being used. Eight injections were made for each test. The volume injected was always 0.5 c.c. of which 0.25 c.c. was 50% Indian ink, the remaining 0.25 c.c. was either distilled water or saline, for the controls, or different amounts of testis extracts alone or with embryonic extracts from cattle or rat embryos. Since the permeability varies with the region of the skin, particularly between abdomen and the back, the control injections were made into corresponding regions of the other side of the body. The measurements of the black spots were taken 15 hr. after the injections.

EXPERIMENTAL

(1) *The effects of embryonic extracts on body weight and growth of sexual organs*

Experiments on guinea-pigs. Kaufman [1932] and Masselin, Julio & Sopena [1934] have shown that injections of embryonic extracts into mice do not influence the growth of the animals. We obtained similar results in guinea-pigs which were injected with embryonic extracts but these injections caused a definite inhibition in the development of the male sexual organs. This could not be attributed to the action of homologous or heterologous proteins since the injections of horse or cattle serum had no effect of this kind.

A typical experiment is illustrated in Fig. 1 and Table I. Fifteen guinea-pigs were divided into three groups of five animals each. One group remained untreated; in the second and third group each pig received several injections of 0.2-0.4 c.c. of 10% cattle serum or 10% cattle embryonic extract respectively. The amounts injected during the 20 days of treatment are given in the figure, the total amounts injected into each animal being 2 c.c. It will be seen that in the first week there was no difference in the increase of body weight between the three

groups, but there was a slight retardation of the pigs treated with embryonic extract during the second and at the beginning of the third week. Their final weights, however, were nearly identical (Table I, cols. 2, 3). The growth of the sex organs differed in the three groups. The

TABLE I. Effects of "Embrionina-ISM" and of cattle serum on weight of body and sex organs in male guinea-pigs

Treatment	Final body weights in g.		Final weight of sex organs in mg.				Final length of seminal vesicles in mm.	
	Each	Average	Each	Average	Average calculated for 1000 g. body weight		Each	Average
None	220, 220, 230, 200, 220	218	1360, 1050, 1380, 1200, 1450	1288	5908		37, 32, 32, 35, 46	36.4
Serum	220, 210, 200, 250, 220	220	1000, 1480, 1150, 1900, 1250	1352	6145		32, 43, 37, 42, 31	37.0
"Embrionina-ISM"	200, 160, 240, 250, 212	212	910, 800, 1050, 1250, 1190	1040	4905		32, 34, 35, 35, 36	34.4

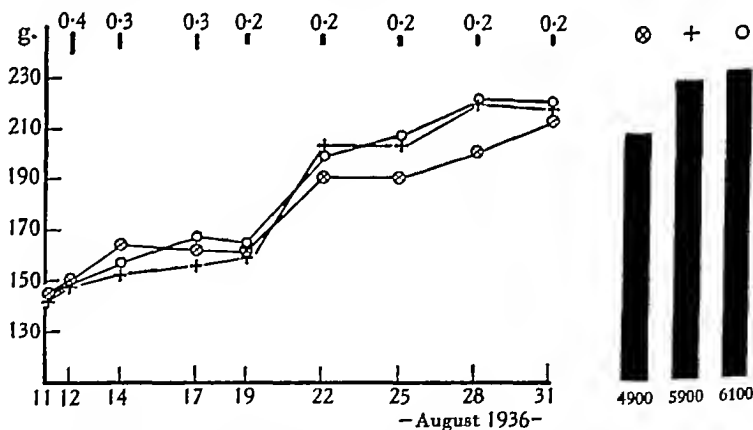


Fig. 1. The curves show the increase in body weight from treated and untreated guinea-pigs. Ordinates: body weight in g.; abscissae: days of the month of August. The columns at the right give the weight of sexual organs in mg. per kg. body weight from the same animals. + = untreated animals; O = animals treated with cattle serum; X = animals treated with "Embrionina-ISM". All values are mean values from five animals.

sexual organs (testes, penis, prostate and seminal vesicles) of the animals injected with cattle serum had grown nearly to the same extent as those of the control animals, their average weight being in fact 3.2% more than that of the controls. The sexual organs of the guinea-pigs injected with embryonic extracts were definitely smaller. Their average weight was 17% less than that of the untreated controls and 20.2% less than

that of those treated with cattle serum. Inhibition in growth of the sexual organs was also shown by the small deficiency in length of the seminal vesicles (Table I, cols. 7, 8). In another and similar series of experiments the average weight of the sex organs was 17% less than in the controls. Calculated for 1 kg. guinea-pig the sexual organs of the control animals gave a mean value of 4958 mg., those of the animals treated with embryonic extracts one of 4107 mg. In this series the average weight of the guinea-pig was 130 g. at the beginning and 190 g. at the end of the experiment, and the controls were injected with horse serum.

That foreign proteins are not responsible for the observed action of embryonic extracts is confirmed by experiments with extracts prepared from guinea-pig embryos. Male and female embryos were extracted separately, the male embryonic extract being injected into male, and the female extract into female guinea-pigs. The results obtained on sixteen animals, half of which were used as controls, are given in Table II.

TABLE II. Effects of guinea-pig embryonic extract on weight of body and of sex organs in guinea-pigs

Treatment	Sex	Final weight of sex organs in mg.						Final length of seminal vesicles in mm.	
		Final body weights in g.		Average		Average calculated for 1000 g. body weight			
		Each	Average	Each	Average			Each	Average
None	Male	230, 190, 190, 170	195	1400, 1100, 1000, 850	1088	5574		37, 32, 33, 26	32.0
Embryonic extract	Male	180, 183, 190, 180	183	800, 860, 910, 880	863	4716		28, 30, 30, 34	30.5
None	Female	260, 240, 200, 230	233	800, 420, 560, 590	593	2545		—	—
Embryonic extract	Female	250, 220, 210, 230	228	490, 500, 540, 630	540	2374		—	—

The inhibition in growth of the male sexual organs is nearly of the same order (15.3%) as that observed when cattle embryonic extract is injected. In the female pigs treated with embryonic extracts there was no definite inhibition in the weight of the sexual organs (ovaries, Fallopian tubes, uteri and vagina). The difference between the two groups was only 6.8% (Table II).

The experiments hitherto reported were carried out during the months July to October. In similar experiments done during the winter months the results were less evident. This is probably due to the fact that normal sexual development during this period of the year is reduced. In one series of experiments carried out in January the difference between

the average weight of the male sexual organs of the untreated and treated animals was 6.9% and, if the testes alone were compared, 10.8%. In another series the difference in the average weight of the testis was 10%. These percentage differences are calculated for equal body weights in the same way as in the experiments of Tables I and II. In experiments during the next summer the difference in weight of the sexual organs after a month of treatment was again over 15%.

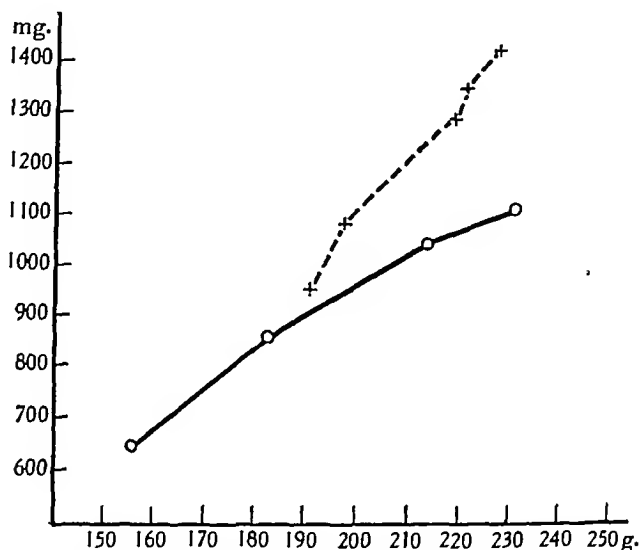


Fig. 2. Relation between body weight and weight of sex organs of male guinea-pigs treated with embryonic extracts (continuous line) and in untreated animals (dotted line). Ordinates: weight of sex organs in mg.; abscissae: body weight in g.

The inhibitory effect on the testes produced by injections of embryonic extracts into guinea-pigs becomes particularly obvious if the weight of the body is plotted against that of the testes. This has been done in Fig. 2 for four series of experiments carried out on forty-five guinea-pigs during the summer months. Each point in the tracing represents the average weight of all normal or treated animals respectively in one series of experiments. The continuous line gives the results obtained from the treated animals, the dotted line those from the controls. The curve obtained from the treated animals does not show the same steep rise as that from the controls; this demonstrates that, with equal body weights, the testes of the treated animals are smaller.

In several experiments the testes of the treated animals were examined histologically (haematoxylin-eosin stain); they had the appearance of normal gonads from young animals. There were no signs of degeneration or any other abnormal changes.

Experiments on rats. The experiments were carried out on fifteen white rats during the months of July and August. In order to find out the relation that existed at the beginning of the experiment between the weight of the body and that of the testis, the left testis was removed in a preliminary operation under ether anaesthesia and weighed. The left testis was chosen because it is usually heavier than the right one, and because some compensatory hypertrophy was to be expected in the remaining testicle. This would increase the normal difference between the weight of the two testes if the right one had been extirpated first, whereas the preliminary extirpation of the larger one tended to diminish this difference. Eight of the fifteen operated rats were given an injection of 0.3 c.c. cattle embryonic extracts on alternate days for 6 weeks. The remaining seven rats were used as controls. In Table III the weights

TABLE III. Effects of "Embrionina-ISM" on weight of body and sex organs in male rats

	Body weight in g.					
	Before		After			
Treatment	Each	Average	Each	Average	Increase Average	
None	180, 150, 120, 90, 60, 170	128.3	180, 170, 140, 110, 80, 170	141.7	13.4	
"Embrionina-ISM"	200, 140, 130, 120, 100, 85, 60, 200	129.4	200, 130, 140, 150, 140, 110, 90, 160	140.0	10.6	
	Weight of testicle in mg.					
	Before		After			
Treatment	Each	Average	Each	Average	Increase Average	
None	1400, 1390, 1350, 1030, 850, 1200	1203	1620, 1700, 1820, 1300, 1300, 1600	1557	354	
"Embrionina-ISM"	1400, 1200, 1200, 1300, 1090, 1180, 550, 850	1096	1420, 1030, 1200, 1500, 1450, 1600, 950, 1100	1281	185	

of the body and of the testis are given before and after the treatment. In order to compare the values obtained it is necessary to express them as ratio of body weights. Taking a body weight of 100 g., the average increase in the weight of the testes of the control animals would be from 937 to 1099 mg., an increase of 17.3 %, and that of the treated rats from 847 to 915 mg., an increase of 8 %, indicating that the growth of the testes in the normal rats was about twice as great as that of the

treated ones. This difference in growth of the testes is particularly significant because the body weight has scarcely increased during the experimental period.

(2) *The effects of embryonic extracts on sexual activity and reproduction*

The experiments were carried out on eighteen male and forty-five female rats and extended over $11\frac{1}{2}$ months. The results are given in Fig. 3 in which the number of rats born per female rat per month are plotted as

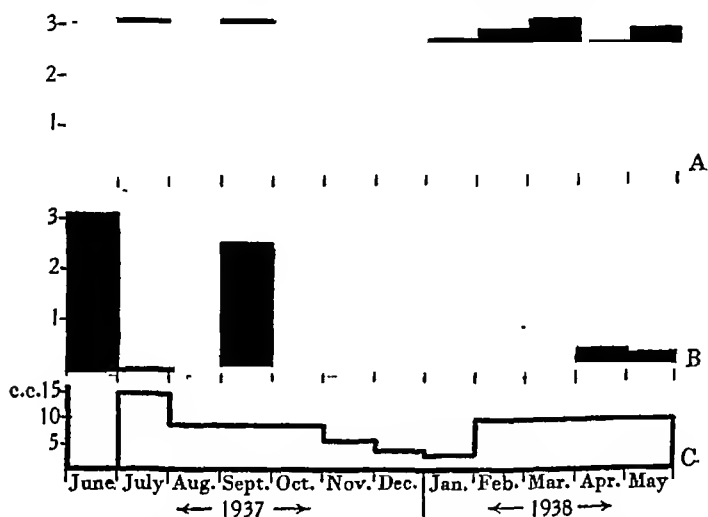


Fig. 3. Effect on fertility of injections of "Embrionina-ISM" into male rats. (A) Control families, (B) families in which the males were treated, (C) dosage of "Embrionina-ISM". Ordinates of A and B: number of rats born per female and per month. Ordinates of C: amounts of "Embrionina-ISM" in c.c. given per month to each male. (For details see text.)

ordinates. The upper part (A) gives the results obtained with the control animals for which nine males and twenty-two females were used, the average number of rats born each month being about three. The middle part (B) shows the number of rats born each month in the series in which the males of each family had been injected with cattle embryonic extract, the injections being given every second day. The amount injected is shown in the lower tracing (C). In the first month of treatment (July) the males and females were kept isolated. When they were reunited it took naturally about a month before any birth could have occurred. There are, therefore, no births in August. In September the number of animals born was 15% less than that of the controls, but in the following

3 months there was cessation of births in all families. The decrease of 15% in September is still within the limit of normal variations, but may already indicate the beginning of the inhibitory effect. With the doses of extracts used for injection, a period of 3 months' treatment appeared to be necessary for the complete development of sterility. The extract was given in diminishing amounts; it will be seen that, after 3 months, births began again. Some families continued to have no new-borns, but in others there were some births. The average number of new-borns per female during this period was 1.25. With renewed increase in the amounts of extract injected the number of births decreased again.

The results obtained in the experiments, in which the female members of the families were injected (Fig. 4) are more difficult to interpret. No cessation of births took place. It is possible that the amount of extract injected was too small and the period of observation too short for the development of the full effect. Furthermore the results obtained on injecting females, who are the passive partners in copulation, cannot be compared with those on injecting males. The frequency of copulation is determined by the male. On the other hand the fact that fertility was not abolished shows that under the conditions of our experiments the extract did not inhibit the function of the female sexual apparatus. There was at first a decrease, then an increase, and finally again a decrease in the number of births.

The injection of embryonic extracts into the females before and during pregnancy did not appear to have any influence on the young. Their weight was the same as that of the new-borns from the control families. The average weight of sixty-three new-borns, the mothers of which had been injected, was 4.98 ± 1.0 g., the value for the controls calculated from 210 new-borns was 5.1 ± 1.2 g. These results do not include premature births. The growth of the new-borns, as determined by the increase in body weight, showed the same behaviour as that observed in the new-borns from the control families, and the mortality of the

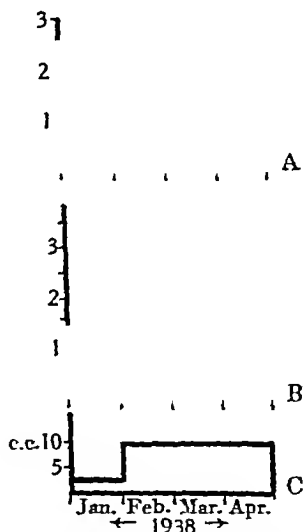


Fig. 4. Effect on fertility of injections of "Embrionina-ISM" into female rats. (A) control families, (B) families in which the females were treated, (C) dosage of "Embrionina-ISM". Ordinates of A and B: number of rats born per female and per month; ordinates of C: amount of "Embrionina-ISM" in c.c. given per month to each female. (For details see text.)

new-borns was the same in all groups, which suggests that the general resistance to infections, etc. had not suffered by the treatment of the mothers.

(3) *The effects of injections of embryonic extracts into rats on the content of R. factor in the testis*

The content of R. factor in the testis varies with the growth of the animal. We found that there was some parallelism between the rate of growth of the testis and its content of R. factor. Testes taken from rats weighing about 90 g., in which the rate of growth of the testis had reached its maximum, yielded the highest value of R. factor. In the later periods of life the value decreased again, and, when the animal had reached a body weight of about 150 g., the value remained approximately constant during the following months. We have, therefore, used for our experiments rats weighing about 150 g. and found that, in these animals, injections of embryonic extracts for a longer period increase the content of R. factor in the testis. For instance, in one rat weighing 150 g. the left testis was removed and used for extraction. It weighed 900 mg. The spreading power of this extract was compared with that of saline solution, the ratio between the two being 1.4 to 1. The rat then received injections of "Embrionina-ISM" for 5 weeks; its weight increased to 170 g. but that of the testis was still 900 mg. after this period as a result of the inhibitory influence of the embryonic extract. When the spreading power of the extract made from the testis was compared with that of a saline injection the ratio between the two was found to be 2.9 to 1. The R. factor content in the testis had therefore been doubled by the treatment.

In other experiments we have tested the spreading power of testicular extracts taken from rats previously treated for 10 months with cattle embryonic extract, and have compared it with that of testicular extracts obtained from normal rats of similar weight. The spreading power of the testicular extracts from the treated animals was approximately twice as great as from the testes of normal rats. This is illustrated in Fig. 5.

In this figure the areas of spread are given by the black columns and the solutions injected are indicated by the rectangles below the columns. The first and fifth columns with areas of spread of 1820 and 1825 mm.² respectively show the effect of the testicular extracts of the treated rats. The testes were extracted with distilled water (first column) or saline (fifth column). The areas of spread of similar extracts prepared from the testes of non-treated rats are given in the ninth and thirteenth columns.

Despite the strong effect which is observed in this experiment with the control tests, giving spreading values of 900 and 1054 mm.², the treatment has led to the usual increase of R. factor content in the testis.

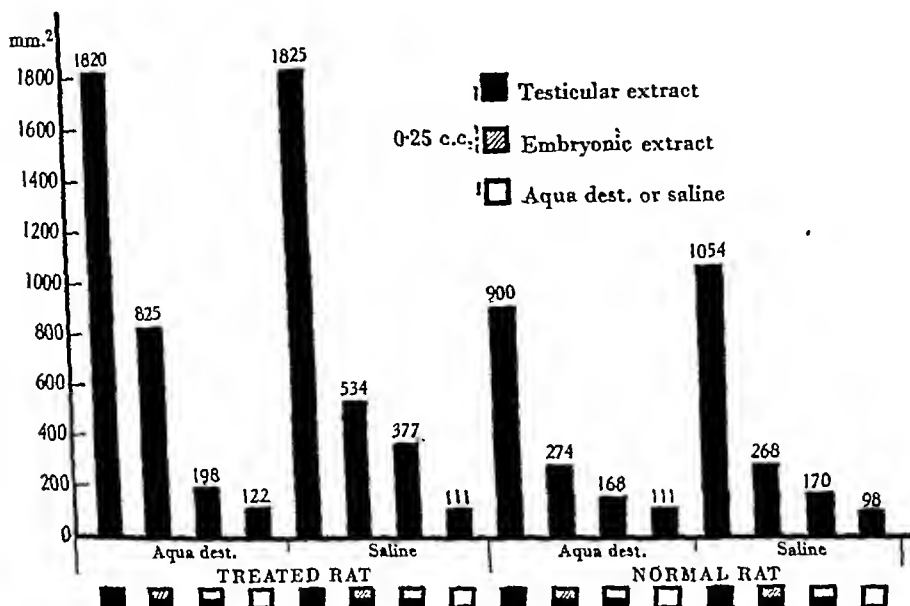


Fig. 5. Effect of embryonic extracts on the spreading power of testicular extracts *in vivo* and *in vitro*. The figures on the left and on top of the columns give the spread area in mm.² (For details see text.)

(4) *The effects of testicular extracts on the spreading power of embryonic extracts in vitro*

The results of the previous paragraph led us to inquire whether embryonic extracts have a similar influence *in vitro* if injected into the rabbit's skin together with testicular extracts.

Extracts obtained from cattle or rat embryos do not contain R. factor. The spreading power of such extracts was in fact smaller than that of distilled water or of saline solution as shown by the experiment in Fig. 6. Embryonic extracts, however, increased the spreading power of testicular extracts when injected together. The effect was not pronounced with testicular extracts



Fig. 6. Spread areas (mm.²) produced in the rabbit's skin by 0.25 c.c. of (A) saline, (B) rats embryonic extract, (C) "Embrionina-ISM".

from horses, the spreading power of which is weaker than that of similarly prepared testicular extracts from rats. The spreading power of these extracts was always greatly augmented when injected with embryonic extracts. The effect was greater when the testes were taken from rats which had been previously treated with embryonic extract. The results were the same when the extracts were prepared with distilled water or with saline solution. In Fig. 5 the spreading power of 0.12 c.c. of testicular extract obtained from rats treated with embryonic extracts for 10 months rose from the values of 198 and 377 (cols. 3, 7) to those of 825 and 534 (cols. 2, 6) when given with embryonic extract. The values for the testicular extracts from normal rats, which were 168 and 170 (cols. 11, 15), rose to 274 and 268 (cols. 10, 14) when tested with embryonic extract. It is unlikely that the specific sexual hormones are involved in this effect since we found that androsterone had no spreading power when injected alone or together with embryonic extract.

When the skin of the test albino rabbit showed signs of skin diseases like psoriasis, the skin reacted irregularly to the various solutions and extracts.

(5) *The oestrogenic activity of the embryonic extracts*

The possibility that some of the effects observed with our embryonic extracts resulted from the presence of folliculin could be excluded. We tested the oestrogenic activity of the extracts of cattle embryos. The folliculin tests in adult castrated mice, as judged by vaginal smears, were always negative, indicating the absence of effective amounts of oestrogenic substances.

DISCUSSION

Our experiments have revealed a hitherto unknown action of embryonic extracts, namely its inhibitory effect on the sex organs, at least on those of male animals. The definite inhibition in the growth of the male sex organs, the cessation of births in rat families, the male members of which had been injected and the absence of any other obvious signs of abnormal development or behaviour in animals treated with embryonic extracts, can only be explained by the presence in these of a principle which has a specific inhibitory influence on the development and function of the gonads. This effect may have been brought about directly by the action of the extracts on the sex organs, or indirectly by the interaction of other endocrine organs or through the central nervous system. We have not determined whether the sterility due to embryonic extracts is produced by an inhibition of sexual activity or by aspermy. Clinical observations (see later) and the histological findings are in favour of the former

mechanism. Making this assumption the inhibition must have been so strong as to produce cessation of sexual activity, since our method of observation would probably not have enabled us to detect partial inhibition only. The facts that the testes of the treated animals resembled normal juvenile gonads histologically and that the sterility stopped when the treatment was discontinued, suggest that embryonic extracts produce, not pathological, but functional changes in the sex organs. We are not able, from our experiments on female animals, to postulate a similar inhibitory action of embryonic extracts on the female sex apparatus. Our experiments on females were probably not carried out for long enough time to allow the full development of such an effect.

The active principle in the embryonic extracts has not been identified, but we can definitely exclude oestrogenic substances. Using our own extracts we may conclude that the active principle is filterable through a Chamberland candle, soluble in water and saline but insoluble in ether. The active principle is not "species specific" and unspecific proteins are not responsible for the effect. We do not know whether the effects of embryonic extracts in sensitizing the spreading power and in increasing the R. factor result from the same principle which produces the inhibitory effects on the male sexual organs.

Our observations naturally led to the question whether embryonic extracts may be used therapeutically in patients with symptoms of sexual hyperexcitation. The results of the clinical trials which will be published in detail elsewhere showed that subcutaneous injections of "Embrionina-ISM" (1-6 c.c. daily) had a pronounced effect in patients sexually hyperexcited.

SUMMARY

1. Subcutaneous injections into guinea-pigs and rats of saline extracts from cattle or guinea-pig embryos inhibited the growth of the testes but did not inhibit the general growth and development of the animals. There was no obvious inhibition of the growth of the female sexual organs.

2. Subcutaneous injections of embryonic extracts into male rats inhibited sexual activity; if the rats were brought together with females there were no births. When the injections were suspended sexual activity returned. Injections of embryonic extracts into the females produced irregular effects on fertility.

3. Subcutaneous injections of embryonic extracts into rats increased the content of R. factor in the testis.

4. Embryonic extracts contained no spreading power but increased the spreading effect of testicular extracts of rats if injected simultaneously into the rabbit's skin. The specific sexual hormones appear not to be involved in this action since the injection of embryonic extract with androsterone had no spreading effect.

5. The embryonic extracts used did not contain oestrogenic substances in detectable amounts.

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THE ABSORPTION OF MONOSACCHARIDES FROM THE DISTAL SMALL INTESTINE OF ANAESTHETIZED CATS

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It is accepted to-day that different sugars are absorbed at different rates from the gut, in general the hexoses being more rapidly absorbed than the pentoses. However, review of the original papers shows that various animals and different techniques have been used with results not altogether in agreement (Table I).

The much more rapid absorption of glucose as compared with xylose in the rat, the observation that this difference disappears in rats poisoned with monoiodoacetic acid, the fact that xylose, in contrast to glucose, is not known to undergo phosphorylation *in vivo*, and other evidence, led Verzár and his co-workers to postulate phosphorylation within the mucous membrane of the gut as the specific process responsible for the preferential absorption of glucose [Verzár & McDougall, 1936].

The validity of this concept has been questioned by Westenbrink [1936b] and by Klinghoffer [1938] chiefly on the grounds that monoiodoacetic acid is certainly not a direct specific inhibitor of phosphorylation and probably is merely a general systemic poison inhibiting indifferently the absorption of glucose, xylose and sodium chloride. Nevertheless, Verzár's hypothesis has been generally accepted and, moreover, regarded as applicable to absorption of glucose in all types of animals. If it can be shown, however, that xylose is absorbed as rapidly as the larger molecule of glucose in any one animal species, then support for the universal applicability of Verzár's hypothesis is very definitely weakened.

Surprisingly enough, McDougall & Verzár [1935] state that "in Katzen-darm scheint Xylose nicht wesentlich langsamer als Glucose resorbiert zu werden". However, they did not pursue the matter further, since cats did not lend themselves to work on absorption, although later, Issekutz, Laszt & Verzár [1938] found that the proximal jejunum of cats absorbed twice as much glucose as xylose from a *mixture* of both sugars.

TABLE I. Relative rates of absorption of monosaccharides from the small intestine.

Author	Animals	Relative rates of absorption
Höber [1899]	Dogs anaesthetized	Galactose = glucose
Hédon [1900]	Rabbits	Glucose > galactose > arabinose > raffinose
Nagano [1902]	Dogs unanaesthetized	Galactose > glucose > fructose > mannose > xylose > arabinose*
Hewitt [1924]	Rabbits un- anaesthetized	Glucose > fructose
	Cats pithed	Glucose > galactose > fructose
Cori [1925]	Rats unanaesthetized	Galactose > glucose > fructose > mannose > xylose > arabinose
McCance & Madders [1930]	Man and rat un- anaesthetized	Xylose > arabinose > rhamnose
Auchinachie, Macleod & Magee [1930]	Rabbits isolated loops	Glucose > xylose
Macleod, Magee & Purves [1930]	Rabbit and cat isolated loops	Glucose > xylose
	Rats unanaesthetized	
Miller & Lewis [1932]	Rats unanaesthetized	Glucose > xylose
Wilbrandt & Laszt [1933]	Rats "Numal" and urethane	Galactose > glucose > fructose > pentoses
Westenbrink [1936b]	Rats unanaesthetized	Galactose > glucose > fructose > mannose > xylose > arabinose
	Pigeons urethane	Galactose > glucose > fructose > mannose > xylose† > arabinose = xylose†
Westenbrink & Gratama [1937]	Frogs anaesthetized	Galactose > glucose > mannose > fructose > xylose > arabinose
Groen [1937]	Man unanaesthetized	Galactose > glucose > fructose
Klinghoffer [1938]	Rats unanaesthetized	Glucose > xylose
Davidson & Garry [1939]	Rats urethane	Galactose > glucose > fructose > xylose

* Comparison made with sugars of very different concentration.

† Naturally occurring *d*-xylose (formerly known as *l*-xylose) positive rotation.

‡ Negative rotation.

In the course of work on the behaviour of monosaccharides in the colon of cats, which animals, incidentally, have rarely been used in work of this type, we controlled our technique by finding the absorption rates for galactose, glucose, fructose and xylose in the small intestine.

At the same time we took the opportunity to find if there was any difference in the rates of absorption of glucose in the distal two quarters of the small intestine. There is a general belief that the rate of absorption of glucose decreases rapidly and progressively towards the caudal end of the small gut. This is supported by Röhmman & Nagano [1903], von Frey [1909], Omi [1909], Westenbrink [1936*a*], Verzár & Wirz [1937], and Lium & Florey [1939], but denied by London & Polowzowa [1906, 1908].

METHODS

Since the cats were almost invariably infected with tapeworms, they were treated with powdered areca nut when first received. They were then placed on a diet of diluted milk for at least 1 week and were fasted

18 hr. before use. Early experiments were performed under urethane anaesthesia. The majority, however, were carried out under the much more convenient anaesthetic "Pernocton" (sodium salt of β -bromallyl-sec.-butyl-malonyl urea). The dose was 70 mg./kg. subcutaneously. The results were similar to those obtained under urethane anaesthesia.

The abdomen was opened in the mid-line and ligatures of narrow tape, which did not cut into the gut wall, tied round the ileum just proximal to the ileocolic sphincter, and more cranial so as to tie off two segments of the small intestine of approximately the same length (25 cm. each). Such segments were each roughly $\frac{1}{4}$ of the entire length of the small intestine. Loose ligatures were placed at the ends of the segments which were then opened at each end and washed out with Ringer's solution at 38° C. The loops were almost invariably empty and little washing was required. The caudal loose ligatures were then tied. From pipettes, calibrated between two marks, 10 c.c. of one sugar solution were run into one segment and 10 c.c. of another sugar solution into the other segment. The segments were then tied off, the abdomen closed and the animal left in warm surroundings for 1½ hr.

The cat was killed by bleeding and the entire small intestine excised. Each loop with its sugar solution was slit open along its whole length over a large filter funnel draining into a 300 c.c. graduated flask. The mucous and peritoneal surfaces were then thoroughly washed with a stream of warm distilled water. The flasks were cooled, the contents made up to the mark and the sugars estimated in duplicate by the method of Hagedorn and Jensen. In many cases additional estimations were carried out by Bertrand's method with satisfactory agreement.

We recovered the sugars quantitatively by this method from loops of gut in dead cats.

The empty loops and the remaining portion of the small intestine were weighed.

The blood pressure was measured at the end of the absorption period, being usually in the neighbourhood of 100 mm. Hg. At the same time arterial blood was taken for blood-sugar estimation. The level was variable but seemed to have no obvious influence on the degree of sugar absorption.

The sugars used were *d*-glucose, *d*-galactose, *d*-fructose and *d*-xylose ($[\alpha]_D + 19.5^\circ$) supplied by British Drug Houses. Solutions were made at least 24 hr. before use.

RESULTS

In the rat we found previously that, after $1\frac{1}{2}$ hr., the loop containing glucose was usually completely collapsed whereas the loop containing xylose was still obviously distended. In the cat, on the other hand, neither loop was collapsed after $1\frac{1}{2}$ hr. although it was obvious that some of the contents had disappeared, and the loops showed a similar degree of distension. This was in agreement with McDougall & Verzár [1935], who found that glucose is not so rapidly absorbed from the intestine of the cat as from that of the rat.

Table II shows the results obtained with glucose and xylose in twelve cats. In six of these glucose was in the upper loop and xylose in the lower. In the remaining six the relative positions of the solutions were reversed.

TABLE II. Absorption in mg. of glucose and xylose during 90 min. from the distal ileum of the cat. Initial amount of glucose, 540 mg. in 10 c.c.; initial amount of xylose, 450 mg. in 10 c.c.

Cat	Glucose		Xylose	
	Total amount absorbed, mg.	mg. absorbed per g. gut	Total amount absorbed, mg.	mg. absorbed per g. gut
10	306	10.2 U	236	8.4 L
11	140	6.7 L	158	7.5 U
12	182	11.4 L	202	11.2 U
24	198	10.5 U	243	9.0 L
25	219	9.5 L	252	11.0 U
26	108	4.3 U	63	2.7 L
27	168	7.6 L	193	9.2 U
28	231	8.9 U	177	6.3 L
29	192	8.0 U	171	7.1 L
30	169	7.4 L	129	5.6 U
31	183	7.3 U	171	8.1 L
32	219	10.0 L	258	11.2 U

Mean, $m_{glu} = 8.56$

Mean, $m_{xyl} = 8.11$

Standard error, $\epsilon_{glu} = \sqrt{\frac{\sum d^2}{n(n-1)}} = 0.5952$

Standard error, $\epsilon_{xyl} = 0.7269$

$$\frac{m_{glu} - m_{xyl}}{\sqrt{(\epsilon_{glu}^2 + \epsilon_{xyl}^2)}} = 0.48.$$

U = cranial loop; L = caudal loop.

The results are expressed as mg. sugar absorbed per g. gut, since we, at least, found it quite impossible to ensure that the segments were of exactly the same length, and it seems justifiable to assume that the absorbing surface must to some extent be a function of the weight of gut in any one animal.

Since we thought it more important to have isotonicity and equal volumes than to have equal weights of sugar, each loop contained the

same volume (10 c.c.) of isotonic sugar solution, 5.4% in the case of glucose and the other hexoses, and 4.5% in the case of xylose. As a result the actual amount of xylose available for absorption was only 450 mg. as compared with 540 mg. of the hexoses. In spite of the smaller amount of xylose available for absorption, even from inspection it was obvious that there was no consistent difference between the amounts of glucose and xylose absorbed per g. gut. The formula recommended by Burn [1937], $\frac{r_1 - r_2}{\sqrt{(e_1^2 + e_2^2)}}$, shows that the difference is definitely not significant (Table II).

Although there was no significant difference between the rates of absorption of glucose and xylose, galactose was significantly more rapidly absorbed than glucose (Table III). On the other hand, fructose was absorbed at roughly half the rate of glucose (Table IV).

TABLE III. Absorption in mg. of glucose and galactose during 90 min. from the distal ileum of the cat. Initial amount of glucose, 540 mg. in 10 c.c.; initial amount of galactose, 540 mg. in 10 c.c.

Cat	Glucose		Galactose	
	Total amount absorbed, mg.	mg. absorbed per g. gut	Total amount absorbed, mg.	mg. absorbed per g. gut
19	316	10.2 L	408	12.0 U
20	463	14.0 U	502	15.2 L
21	231	12.1 U	270	13.5 L
22	249	13.1 L	411	19.5 U
23	264	11.5 U	290	15.2 L

Mean, $r_{glc} = 12.18$

Standard error, $e_{glc} = 0.6338$

Mean, $r_{gal} = 15.08$

Standard error, $e_{gal} = 1.2560$

$$\frac{r_{gal} - r_{glc}}{\sqrt{(e_{gal}^2 + e_{glc}^2)}} = 2.05.$$

U = cranial loop; L = caudal loop.

TABLE IV. Absorption in mg. of glucose and fructose during 90 min. from the distal ileum of the cat. Initial amount of glucose, 540 mg. in 10 c.c.; initial amount of fructose, 540 mg. in 10 c.c.

Cat	Glucose		Fructose	
	Total amount absorbed, mg.	mg. absorbed per g. gut	Total amount absorbed, mg.	mg. absorbed per g. gut
13	153	6.2 U	80	4.0 L
14	132	5.7 L	64	3.2 U
15	228	8.1 U	82	3.6 L
16	266	11.6 L	106	4.4 U
18	183	7.5 L	106	4.2 U

Mean, $r_{glc} = 7.82$

Standard error, $e_{glc} = 1.0399$

Mean, $r_{fru} = 3.88$

Standard error, $e_{fru} = 0.2154$

$$\frac{r_{glc} - r_{fru}}{\sqrt{(e_{glc}^2 + e_{fru}^2)}} = 3.71.$$

U = cranial loop; L = caudal loop.

Incidentally, the difference in the means for the absorption rates of glucose in the three series shows the importance of carrying out such comparisons in the same animal.

The data for glucose absorption from the above three groups, along with figures from three additional cats, were then separated into two classes according to the positions of the loops. The mean absorption from thirteen upper loops was 8.8 mg./g. gut, and that from twelve lower loops 9.2 mg./g. gut. Although the comparisons were not carried out on the same animals, yet there is no evidence here for decreasing powers of absorption in the more caudal portion of the region of the gut used by us.

DISCUSSION

All such experiments as the present are open to the objection that the animals are in an abnormal state since they are anaesthetized. There seems little doubt that cats, under such different anaesthetics as chloralose and "Numal" [McDougall & Verzář, 1935], and urethane and "Pernoxon" as in the present series, do not show preferential absorption of the hexose glucose as opposed to the pentose xylose. In spite of this, under exactly the same conditions, glucose is absorbed more slowly than galactose and much more rapidly than fructose. It thus seems to be unlikely that the animals are in such poor condition that selective absorption cannot take place. Incidentally, in rats under urethane anaesthesia, with similar technique, we found an obviously preferential absorption of glucose as compared with xylose in the distal ileum [Davidson & Garry, 1939]. To make assurance doubly sure, we subsequently carried out experiments on rats, using the two loop technique in the distal ileum, with the same sample of xylose which we used in the cats, and found the usual marked preferential absorption of glucose.

It is stated that preferential absorption of glucose in the rat is more marked in the cranial regions of the small intestine [Verzář & Wirz, 1937]. Thus it could be argued that our failure to show similar results in the cat was due to our use of the distal portions of the gut only. However, even the distal ileum of the rat shows such preferential absorption of glucose [Davidson & Garry, 1939], and the distal ileum of our cats showed marked differences in the rates of absorption of the three hexoses. Moreover, McDougall & Verzář's [1935] own observations on cats ought not to be neglected. It thus seems to be impossible to escape the conclusion that certain absorptive processes are not the same in the cat and rat.

While it cannot be denied that glucose may be absorbed by a selective process, it is unfortunate that the theory of the mechanism involved should rest to a large extent on a difference in rate of absorption of glucose and xylose. Examination of Table I shows that satisfactory evidence of such a happening was obtained mainly in the rat. It would appear that results obtained in one species of animal cannot be automatically regarded as valid for another species, and that no hypothesis of general application dare be based on results from only one or two types of animals.

We cannot say whether or not there is a marked difference between the rates of absorption of glucose in the cranial and caudal regions of the small intestine of cats, but, at any rate, the absorbing powers in the ileum are far from negligible, and there is in that region no sign of an obvious gradient.

SUMMARY

In cats, anaesthetized with urethane and "Pernocton", xylose is absorbed as rapidly as glucose from the caudal region of the small intestine.

In this respect the cat differs from the rat.

Galactose appears to be absorbed more rapidly and fructose more slowly than glucose.

The bearing of these findings on the theory of the mechanism of preferential absorption of glucose is discussed.

Within the caudal half of the small intestine of the cat there is no evidence for decreasing absorptive power for glucose in the more distal region.

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PROLONGATION OF PREGNANCY IN THE HYPOPHYSECTOMIZED RABBIT BY PROGESTERONE AND OESTROGENS

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IN a previous communication [Robson, 1939] it was shown that, when rabbits were hypophysectomized during pregnancy, abortion which otherwise follows within 24-48 hr. could be prevented by the administration of oestradiol benzoate. When injection of the oestrogen was discontinued on the 29th day of pregnancy some of the animals expelled normal live foetuses 2-3 days later.

As previous work has also provided evidence for the view that the luteal function is under the control of an oestrogen, and since it is known that the abortion which follows hypophysectomy is due to the cessation of the luteal function, it was concluded that the oestrogen maintained the pregnancy in hypophysectomized rabbits by virtue of its action on the luteal secretion.

Since the injection of oestrogen in the hypophysectomized rabbit was capable of maintaining pregnancy up to full term, and since the cessation of the injections was followed by a cessation of the luteal secretion and by the consequent expulsion of the uterine contents, it was expected that the administration of oestrogen beyond full term to hypophysectomized rabbits would result in the maintenance of the luteal function and consequently in a prolongation of pregnancy.

As it had, however, not been determined whether the administration of the luteal hormone would prolong pregnancy in the hypophysectomized rabbit as it does in the normal rabbit [Heckel & Allen, 1939] this question was thus first investigated.

The experiments can therefore be divided into two groups, namely: (1) experiments on the prolongation of pregnancy with progesterone; and (2) prolongation of pregnancy with oestrogens.

METHODS

The mating of rabbits was observed and the duration of pregnancy was thus accurately established. The pituitary was removed by the orbital route [Firor, 1933] on the 28th or the 29th day after mating, and thereafter the animals were weighed and carefully palpated every day to determine the fate of the gestation. The completeness of the pituitary removal was checked at post-mortem by macroscopic examination of the sella.

All animals were injected twice daily, the first injection being given immediately after the operation. The oestrogens and progesterone were always administered subcutaneously in solution in oil of sesame. I am indebted to Dr Neumann of Schering, Ltd., for the supply of progesterone used in these experiments.

RESULTS

The results of the various experiments are collected in Tables I and II.

It will be seen that pregnancy was prolonged in three out of four of the rabbits hypophysectomized on the 28th or 29th day of gestation and thereafter injected with a daily dose of 1.9 or 2.5 mg. of progesterone. In two of the animals injected with the larger dose dead fetuses showing a fairly advanced stage of reabsorption were present in the uterus on the 38th and 39th day respectively after mating, when the animals were killed for post-mortem examination. Progesterone is thus capable of prolonging pregnancy in rabbits hypophysectomized shortly before parturition.

It is of interest that the doses of luteal hormone necessary to maintain pregnancy in rabbits hypophysectomized during the course of gestation [Robson, 1936, 1937*a*] are very similar to the amounts of progesterone needed to prolong pregnancy in animals in which the pituitary is removed just before parturition.

Prolongation of pregnancy was also obtained in the hypophysectomized rabbit by the administration of oestrogens.

In all the animals injected with a daily dose of 10 μ g. of oestrone, or 5 μ g. of oestradiol, the pregnancy was prolonged several days beyond the normal time of parturition, which usually occurs in our stock on the 30th or 31st day after mating. In all but one (918) of the experimental animals, however, the fetuses were expelled before the 36th day of pregnancy. In rabbit 918 post-mortem examination revealed that the fetuses were dead and showed early signs of reabsorption by the 36th day after mating.

TABLE I. Data on the prolongation of pregnancy in hypophysectomized rabbits by progesterone and oestrogens

Animal no.	Wt. in kg.	Day in pregnancy of hypophysectomy	Injections			Post-mortem findings		
			Substance	Daily dose	Period after mating	Days mammary gland matting	Wt. of g.	Hypophysectomy Complete
808	2.0	28	Progesterone	1.0 mg.	28-35	36	29	
						Fate of pregnancy		
						Live foetuses expelled on 30th and 33rd days; dead foetuses expelled on 35th day		
1043	2.2	20	"	2.5 mg.	20-36	39	—	"
						Killed on 30th day. Uterus contains six macerated foetuses		
1063	2.4	20	"	2.5 mg.	20-31	32	—	"
1050	2.0	20	"	2.5 mg.	20-37	38	43	"
						Foetuses born on 31st and 32nd days		
						Killed on 38th day. Uterus contains eight macerated foetuses		
018	2.7	28	Oestrone	10 µg.	28-35	36	28	"
						Killed on 30th day. Uterus contained two macerated foetuses		
021	2.5	28	"	10 µg.	28-35	36	30.5	"
022	2.8	28	"	10 µg.	28-35	36	—	"
029	2.2	28	"	10 µg.	28-34	35	20.5	"
040	2.6	29	Oestradiol	5 µg.	20-34	35	26	"
1008	2.6	29	Oestradiol benzoate	2 µg.	20-37	38	—	"
						Animal died on 38th day. Six macerated foetuses <i>in utero</i> , one in vagina and one aborted		
1009	2.6	20	"	2 µg.	20-35	35	—	"
						Animal died on 35th day. Uterus contained two large normal looking foetuses		
1013	2.6	20	"	2 µg.	20-38	39	30	"
1008	2.5	20	"	2 µg.	20-36	36	—	"
1110	2.2	20	"	2 µg.	20-37	38	—	"
043	2.5	20	Triphenyl ethylene	20 mg.	20-33	34	—	"
						All foetuses expelled dead on 32nd-34th days		
045	2.4	20	"	40 mg.	20-37	37	24	"
						Killed on 37th day. Uterus contained five macerated foetuses		

As it has been previously found [Robson, 1939] that oestradiol benzoate is more effective than oestrone in maintaining pregnancy in hypophysectomized rabbits, the effectiveness of the former compound in prolonging pregnancy was then investigated. It was found that gestation was prolonged in all five rabbits hypophysectomized on the 29th day of pregnancy and then injected with a daily dose of $2\mu\text{g.}$ of oestradiol benzoate, and that, moreover, expulsion of dead and re-absorbing foetuses occurred at a later stage than in those animals injected with oestrone or oestradiol.

In two further experiments the synthetic oestrogen triphenyl ethylene was injected in animals hypophysectomized just before parturition in order to determine whether it would have the same effect in prolonging pregnancy as natural oestrogens. It was found that a daily dose of 20 mg. of triphenyl ethylene produced only a slight prolongation of the gestation, whereas 40 mg. per day produced a prolongation equal to that brought about by the daily administration of $2\mu\text{g.}$ of oestradiol benzoate.

In order to eliminate the possibility that the administration of oestrogen would prolong pregnancy in the absence of the corpus luteum, a control experiment was performed: both ovaries as well as the pituitary were removed in one rabbit (1023) which then received daily doses of $2\mu\text{g.}$ of oestradiol benzoate. It was found that pregnancy was not prolonged, and that expulsion of the uterine contents occurred 2 days after the operation, i.e. on the 31st day after mating.

TABLE II. Data of experiments on the prolongation of pregnancy in normal rabbits with oestrogens

Animal no.	Wt. in kg.	Injections			Fate of pregnancy
		Substance	Daily dose $\mu\text{g.}$	Period after mating	
923	2.5	Oestrone	10	28-34	All foetuses expelled dead on 33rd-34th days
928	1.7	"	10	28-34	Killed on 35th day. Uterus contained four dead foetuses, one aborted on 35th day
964	3.3	Oestradiol benzoate	2	29-31	Live litter on 31st-32nd days
1010	2.5	"	2	29-33	Live foetuses expelled on 33rd-34th days
1011	3.0	"	2	28-32	Live foetuses expelled on 32nd-33rd days
1012	2.6	"	2	29-34	All foetuses expelled dead on 34th day

A further series of experiments was performed in order to establish what doses of oestrogen are necessary to prolong pregnancy in the normal (as shown by Heckel & Allen, 1939) as compared with the hypophysectomized rabbit. The results are given in Table II. It will be seen that in none of the six animals which received a daily dose of either 10 μ g. of oestrone or 2 μ g. of oestradiol benzoate was pregnancy prolonged beyond the 35th day. In three of these animals indeed, live litters were born at, or just beyond, the normal time. It would thus seem that the doses of oestrogen necessary to prolong pregnancy in the rabbit are greater in the normal than in the hypophysectomized animal.

Examination of the ovaries in the animals of the various experimental groups showed that the prolongation of pregnancy by oestrogens is accompanied by a maintenance of the luteal structure present at the time of the pituitary removal. This stands in contrast to the results obtained in pregnant hypophysectomized rabbits not treated with oestrogens, in which the corpora lutea rapidly degenerate and in which the cessation of the luteal function is followed by expulsion of the uterine contents. The condition of the corpus luteum in a hypophysectomized rabbit (1008) in which pregnancy was prolonged to the 38th day after mating by the administration of an oestrogen is shown in Pl. I, fig. 1.

When pregnancy was prolonged in the hypophysectomized rabbit by means of progesterone, it was found that the corpora lutea showed some degree of degeneration as compared with the corpora lutea in those animals in which prolongation of pregnancy was produced by the administration of an oestrogen. At the same time the degree of degeneration of the corpus luteum did not appear to be quite as marked as when the gestation is not maintained by the administration of progesterone. These results are of interest in view of the previous findings on the condition of corpora lutea in rabbits hypophysectomized *during* gestation and injected with the luteal hormone [Robson, 1936]. In these animals, too, the corpora lutea did not degenerate as they would have if abortion had taken place in the absence of any injections of progesterone.

These results may be explained on the assumption that the uterine contents secrete small quantities of oestrogen which have some action in maintaining the structure of the corpus luteum in hypophysectomized rabbits in which pregnancy is maintained or prolonged by progesterone.

DISCUSSION

There is much evidence that in the rabbit the end of pregnancy and onset of parturition are dependent on a cessation of the secretion of progesterone by the corpus luteum. This is shown by many experiments on the effects of removal of the ovaries during pregnancy, and of the injection of the luteal hormone into ovariectomized pregnant animals. Further evidence is provided by the results obtained when the luteal activity is experimentally prolonged beyond the normal time. Thus, if at the end of pregnancy progesterone is injected in order to maintain the level of the luteal hormone which exists during the course of gestation, the pregnancy is prolonged and this occurs whether the pituitary gland is present, as previously shown by Heckel & Allen [1938], or whether, as demonstrated by the present experiments on hypophysectomized animals, the pituitary is absent. Moreover, prolongation of pregnancy is also produced by inducing the formation of new corpora lutea during the later stages of pregnancy by the intravenous injection of gonadotrophic hormone [Snyder, 1934] and thereby continuing the period of luteal activity.

The question then arises, what is the mechanism responsible for the cessation of the luteal function at the end of the normal gestation? It appears probable that the luteal function is ultimately controlled by the anterior pituitary, and it is therefore likely that some change in the secretory activity of the hypophysis occurs during the later phases of pregnancy.

But recent evidence is tending to show that the factor which is directly responsible for the control of the luteal secretion is an oestrogen [Robson, 1937*b*, 1938; Westman & Jacobson, 1937]. This view is based on the finding that oestrogens will maintain the luteal function in the hypophysectomized pseudo-pregnant and pregnant rabbit, and that in the latter this actually results in the maintenance of gestation. The present experiments give further support to this view by showing that if the luteal secretion is maintained beyond full term by the administration of oestrogen, parturition does not occur and pregnancy is prolonged.

In previous experiments it was found that the doses of oestradiol benzoate needed to maintain the luteal function and pregnancy in the rabbit hypophysectomized on the 21st day of gestation were of the order of 1.5–2 μ g. per day. The present experiments show that the same amount of oestradiol benzoate per day, i.e. 2 μ g., will prolong the luteal function beyond its normal time and thereby produce a prolongation of gestation.

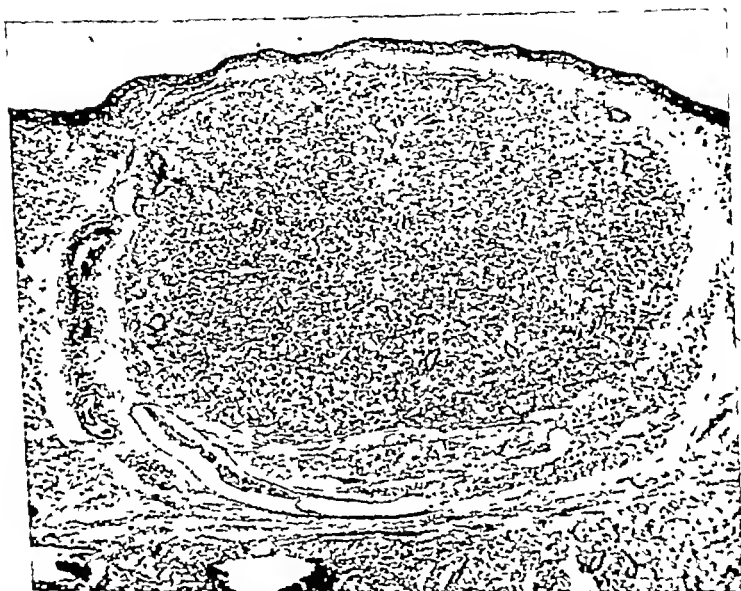


Fig. 1. Microphotograph of a corpus luteum in a rabbit hypophysectomized on the 29th day of pregnancy and thereafter injected with 2 μ g. of oestradiol benzoate. Pregnancy was prolonged and the animal was killed on the 38th day after mating. $\times 40$.

These results show that the response of the corpus luteum to oestrogen does not undergo any appreciable change at the time of parturition, and that the cessation of the luteal function is therefore presumably not due to the corpus luteum becoming refractory to the control exercised over it by oestrogen.

Hence, if it be accepted that oestrogen is the actual factor which directly controls the luteal function during pregnancy in the rabbit, it follows that the cessation of the luteal function which precedes parturition must be due to a fall in the effective concentration of oestrogen in the body.

It is to be presumed that the level of oestrogen is in its turn controlled by the anterior pituitary, and the fall in the oestrogen level which precedes parturition may therefore be due to a decrease in the production of gonadotrophic hormone. In this connexion the findings of Hill [1934] are of interest, since he showed that the gonadotrophic hormone content of the rabbit's pituitary reaches a maximum at about the 25th day of pregnancy and decreases thereafter. What happens to the hormone content of the pituitary and to its secretory activity when new corpora lutea are produced experimentally during pregnancy by the injection of gonadotrophic hormone is a matter for further investigation.

The present experiments also raise the question why threshold doses of oestrogen are more effective in prolonging pregnancy in hypophysectomized than in normal rabbits. Several explanations present themselves, but the following appears to be the most likely. The operation of hypophysectomy by the orbital route involves removal of the posterior as well as the anterior lobe of the gland. The production of oxytocin is presumably thereby decreased or abolished, and it seems reasonable to assume that parturition can more easily be inhibited under such conditions than when the posterior lobe is present and exerts its normal secretory activity.

SUMMARY

1. Pregnancy can be prolonged in rabbits hypophysectomized on the 28th-29th days after mating by the administration of (1) 1.9-2.5 mg. of progesterone per day, (2) 2 μ g. of oestradiol benzoate per day, (3) the synthetic oestrogen, triphenyl ethylene. The action of oestrogen is very probably due to a maintenance of the activity of the corpus luteum.

2. In the normal rabbit the daily administration of 2 μ g. of oestradiol benzoate from the 29th day of pregnancy is less effective in prolonging pregnancy than in the hypophysectomized rabbit.

3. The relevance of these findings to the function of oestrogen and the corpus luteum in the maintenance of normal pregnancy is discussed.

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RESPIRATORY REFLEXES IN THE FOWL

By J. D. P. GRAHAM

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HERING & BREUER [1868] described the respiratory reflex in mammals whereby the stimulation of alternate inflation and deflation of the lungs gives rise to impulses in the vagi which result in the inhibition of inspiration and expiration. In this way the respiratory rate is kept automatically at an accelerated rhythm. This work was confirmed by Loewy [1888] and Head [1889] and the final links in the chain of evidence were supplied by Adrian [1933] and Gaylor [1934].

Further, Lumsden [1924] described a reflex in the mammal whereby the passage of a stream of air in the trachea initiated a series of impulses which cut short respiration in a manner closely resembling the Hering-Breuer reflex.

The present paper gives details of an investigation into the presence and mode of working of these reflexes in birds, as represented by the fowl.

METHODS

The presence of the Hering-Breuer reflex was investigated by the following methods in the fowl anaesthetized with nembutal (0.75 grains intramuscularly per kilo weight).

(a) The fowl was laid supine and the vagus nerves isolated. Respiration was recorded with a stethographic lever [Bell & Smellie, 1933]. Both vagi were cut.

(b) With the fowl supine one vagus nerve was isolated. Respiration was recorded by a stethographic lever. The isolated vagus was then cut. The central end was stimulated with various strengths of induced current. Several preparations were used so that the effect of cutting the left and right vagus could be determined.

(c) The fowl was supine and part of the trachea was freed. The trachea was clamped at full inspiration or at full expiration and the effect noted.

(d) The thorax was opened and the right mesobronchus clamped. The thorax was then closed. This technique has been more fully described

[Graham, 1940]. The respiration was recorded by a stethographic lever. A hollow needle (a lumbar puncture needle of 1.4 mm. diameter) was thrust into the right abdominal air-sac. The needle was attached by pressure tubing to a recording mercury manometer arranged to write on the kymograph below the stethographic lever, and to a hand pump. By this procedure the right lung and its appendages, the air-sacs, were isolated with their nerve supply intact, and the air within them stabilized

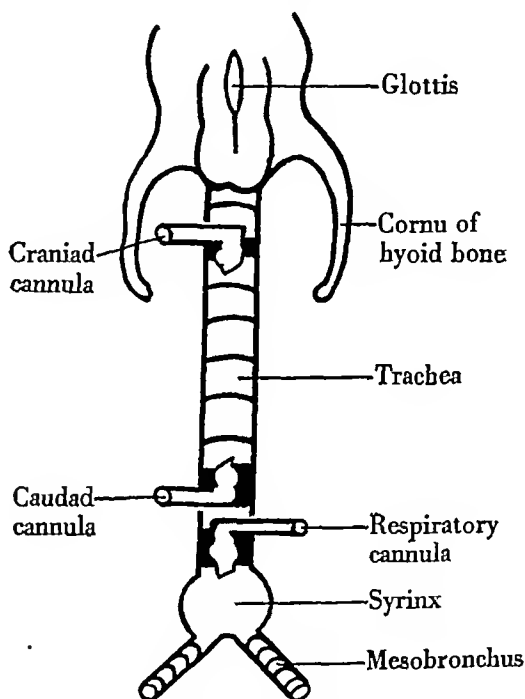


Fig. 1. Diagram of upper respiratory tract of fowl, showing cannulae in position for investigation of tracheal reflex.

at atmospheric pressure. The pressure was increased by pumping or reduced by applying suction and the effect on respiration recorded, together with the altered pressure level. The experiment was modified by section of the right vagus nerve or of both vagi.

(e) The tracheal reflex described by Lumsden [1924] was investigated by isolating as long a portion of the trachea as possible, and inserting three cannulae into it in the manner shown in Fig. 1. The cannula placed low down in the neck (Fig. 1) served for the continuance of respiration, which was recorded by a stethograph level applied to the carina. Of the

other two cannulae, one was placed as high up as possible, facing caudad, the other was put as low down as possible, facing away from the lungs (craniad). A hand pump could be attached to either of these cannulae in order to send a stream of air along the isolated portion of trachea to imitate the inspiratory or expiratory flow. The duration of passage of such an air stream was recorded by a signal lever on the kymograph. This experiment was modified by single or double vagotomy and by the application of a local anaesthetic to the mucous membrane of the trachea.

RESULTS

(a) Respiration in the anaesthetized fowl is regular in rate and rhythm. Severance of the trunks of the vagi in the neck, after a short

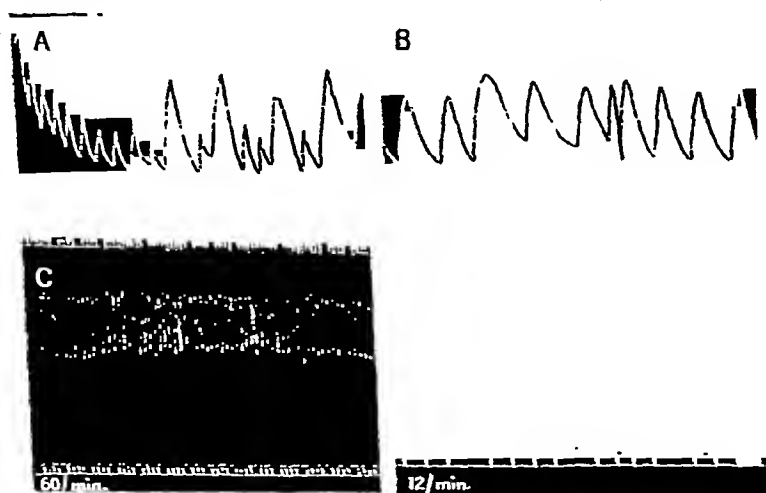


Fig. 2. Fowl, 2.4 kg. Nembutal 0.75 grains per kg. body weight intramuscularly. Respiratory records taken by stethograph lever. Read from left to right, inspiration upwards. C. Normal breathing. A. 2 min. after section of both vagi in the neck. B. 2.8 kg., anaesthetic and record as above. Effect of stimulation of central end of cut right vagus, left vagus intact, induction coil at 20 cm.

period of increased inspiratory tonus, slows the rate of respiration from 44 to 18 per min. (average of ten experiments), and increases the depth of respiration as judged by the stethograph. This alteration of the rate and rhythm is clearly seen in Fig. 2A.

(b) The effect of section of one vagus nerve in the neck is to render the respiration somewhat irregular and slow. Stimulation by induced currents of the central end of either vagus nerve, with the other nerve intact, produces slowing of the respiratory movements, increased in-

spiratory tonus and prolongation of expiration (see Fig. 2B). Stronger stimulation tends to cause increased inspiratory tonus with dyspnoea and convulsions.

(c) If the respiratory movements be recorded on a fast drum the effect of occluding the trachea at the end of inspiration or expiration is as follows. In either case clamping of the trachea affects the next respiration. If the trachea be clamped at the height of inspiration this is followed by a short but definite pause before the next inspiration. If the trachea be clamped at the end of expiration the next inspiration follows at once. These phenomena are illustrated in Fig. 3 (A, B), which

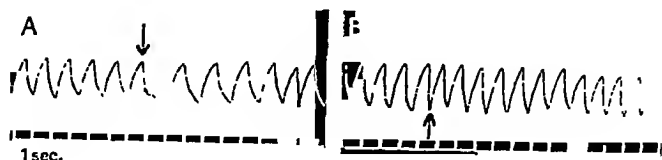


Fig. 3. Fowl, 2.0 kg., nembatal 0.75 grains per kg. intramuscularly. Respiratory record taken by stethograph lever. Read from left to right, inspiration upwards. A. Effect of clamping trachea at full inspiration (marked ↓). B. Effect of clamping trachea at full expiration (marked ↑).

may be compared with Fig. 477 in the current edition of Starling's *Physiology* [Evans, 1936]. There is no evidence of an increase in the height of inspiration following clamping of the trachea, as in the mammal.

(d) The anaesthetized fowl is able to maintain satisfactory respiration after clamping the right mesobronchus. The rate is considerably increased and the amplitude diminished. The respiratory movements are regular in rate and rhythm. On inflating the lung and air-sacs on the right side the carina is raised by the filling of the thoracic and interclavicular air-sacs. This effect modifies the record of respiration, giving a fallacious impression of increased inspiratory tonus. Superimposed upon this alteration in the respiratory record is the true effect of increased pressure in the lung, viz. marked expiratory effort followed by inhibition of respiration as a whole. This effect is shown in Fig. 4A. In this preparation, section of the right vagus nerve in the neck, or of both vagi, abolishes this expiratory effort and respiration assumes the slow rhythm seen after vagotomy. The mechanical raising of the carina is unaffected. The application of suction to the isolated lung system has no effect on respiration. The form of the record resulting from mechanical distension

of the lung system in which the bronchus has been clipped is seen in Fig. 4B, where the pressure in the right lung has been increased after death. This record may be taken as a base line when considering the alteration in respiration due to changes in pressure in the lung and air-sacs.

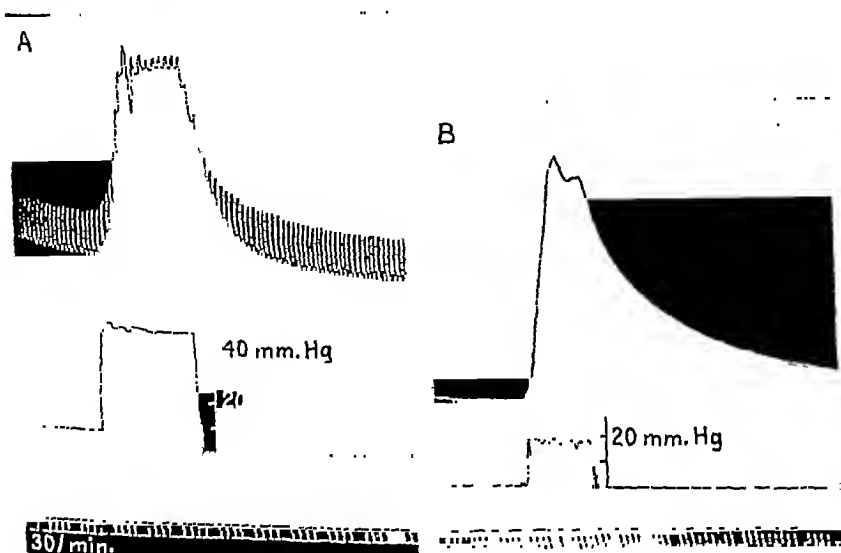


Fig. 4. Fowl 2.5 kg., nembatal 0.75 grains per kg. intramuscularly. Right mesobronchus clamped. Upper record respiration recorded by stethograph lever; lower record pressure in right lung and air-sacs in mm. Hg. Read from left to right, inspiration upwards. A. Effect of raising pressure in isolated lung system. B. Effect of raising pressure in same lung system after death.

(e) If the respiratory movements in an anaesthetized fowl be recorded and the inspiratory air-stream be imitated according to the method described above [Fig. 1, p. 526], the rate of respiration is quickened to a slight degree, but the respiratory movements otherwise remain regular (Fig. 5B). If the stream of air be reversed, as in expiration, the respiratory rate is slowed and expiration prolonged. The expiratory tonus may be increased and if the artificial expiratory stream is begun during inspiration, that inspiration is inhibited markedly (Fig. 5A). If the mucous membrane lining the isolated portion of trachea be swabbed with 2% Decicaine solution, after 5 min. this reflex prolongation of expiration cannot be elicited. The reflex returns after 1 or 2 hr. when the effect of the local anaesthetic has worn off. Careful stripping of all connective tissue from the outer surface of the isolated portion of trachea severs it from its nervous connexions, and also destroys the reflex. The nerve

supply to the trachea of the fowl is from the right and left vagus nerves. Section of the vagi in the neck above the level of the upper tracheal opening abolishes the reflex.

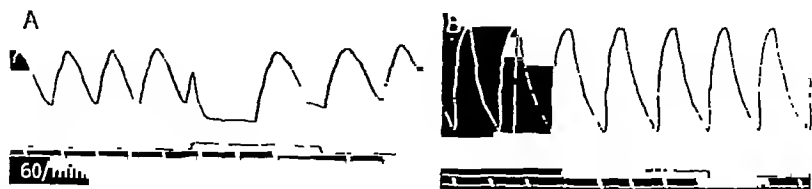


Fig. 5. Fowl, 1.8 kg., nembutal 0.75 grains per kg. intramuscularly. Record of respiration by stethograph lever after triple tracheotomy (see Fig. 1). Read from left to right, inspiration upwards. A. Effect of expiratory air stream in trachea. B. Effect of inspiratory air stream in trachea. Note division of a normal respiratory movement into inspiratory and expiratory phases.

DISCUSSION

That the Hering-Breuer reflex plays a fundamental part in the regulation of respiration in mammals has long been recognized. The various methods of investigating the reflex are set out in the series of classical papers mentioned above. The evidence which has accrued during the present work clearly shows that the Hering-Breuer reflex is present and operates in the fowl. The evidence for this may be briefly recapitulated thus. Section of the vagus nerve slows respiration (after the classical initial stimulation). Impulses which give rise to acceleration of respiration must therefore pass in the vagi. Stimulation of the central end of a cut vagus, the other nerve being intact, may give rise to increased inspiratory tonus or to prolongation of respiration. This reveals the probable nature of the impulses relating to respiration which pass up the vagus nerve. Low air pressure within the lungs, produced by clamping of the trachea at the end of expiration, induces immediate inspiration. Inflation of the isolated lung and air-sacs on one side induces an increased expiratory effort, which, on failure to reduce the intrapulmonary tension, is followed by inhibition of respiration as a whole. Section of the vagus nerves abolishes this effect. From these facts it is inferred that expansion of the avian lungs and air-sacs on inspiration gives rise to a series of impulses which pass up the vagi and cause the inspiration to be cut short. Similarly collapse of the lungs and air-sacs on expiration causes impulses to pass up the vagi which cut short expiration. This is the "Hering-Breuer reflex". It has been held [Thomson, 1923] that in birds inspiration is a passive act following upon forcible expiration. Examina-

tion of the form of the stethographic record of respiration in the anaesthetized fowl lying supine shows that under these circumstances this is not so (see Fig. 5). The lever rises sharply on inspiration and falls slowly on expiration. It is interesting to compare this with the post-mortem record of Fig. 4B. Here the lever rose sharply on inspiration produced by positive ventilation (active), and fell slowly on expiration produced passively by release of the valve of the pump.

In mammals the expiratory impulses in the vagi started by inspiration play a more prominent part in the regulation of respiration than do the inspiratory impulses. This is so in the fowl as positive ventilation results in expiratory effort, while negative ventilation has no effect on respiration; in the experiment where the trachea was clamped, inflation of the lung and air-sacs (full inspiration) had more effect on the respiratory rhythm than deflation of the lung (full expiration).

From the experiments where a stream of air was directed along the trachea while the anaesthetized fowl breathed through a separate tracheotomy tube, it is evident that we have here a reflex peculiar to birds and distinct from Lumsden's variety of the Hering-Breuer reflex. Blowing air along the trachea in the direction of inspiration has no effect on respiration, but blowing air along the trachea in the direction of expiration markedly inhibits inspiration. This is abolished by high section of the vagi nerves or cocainizing of the mucous membrane of the trachea. It is concluded, therefore, that we have here a reflex action by which expiration, once begun, is continued.

In the fowl, therefore, inspiration begins, presumably as a result of increasing carbon dioxide tension in the blood. Distension of the lungs and air-sacs as a result of inspiration acts as a stimulus for the first part of the Hering-Breuer reflex whereby inspiration is inhibited. Expiration begins, largely as a passive movement (in the fowl placed on its back), and the passage of the expired air up the trachea promotes expiration, which continues as a forceful act. That portion of the Hering-Breuer reflex whereby expiration automatically promotes inspiration is weak. Expiration presumably ceases with alteration of blood pH. The unusual feature whereby we find two powerful reflexes inhibiting inspiration and promoting expiration may conjecturally be related to the function of flight in birds and to the peculiarities of respiration during flight, or to the fact that the flow of air during inspiration is an unobstructed one from the trachea to the air-sacs *via* the main bronchi, while the air stream during expiration must meet with considerable obstruction in its passage through the recurrent bronchi and the fine parabronchial air capillaries

where gaseous exchange takes place [Graham, 1940]. Examination of a normal record of respiration shows that expiration lasts twice as long as inspiration (Fig. 5B).

SUMMARY

1. Section of the vagus nerves in the neck of the anaesthetized fowl slows the respiratory movements (after initial stimulation).

2. Stimulation of the central end of one cut vagus with a weak induced current (the other vagus being intact) slows respiration and increases inspiratory tonus.

3. Clamping of the trachea after expiration promotes inspiration and vice versa.

4. Inflation of the isolated lung and air-sacs on one side promotes expiratory movements; deflation of the lung has no effect on respiration.

5. Blowing air in an expiratory manner along the trachea (*in situ* but separate from the lungs) inhibits inspiration. This effect is abolished by section of the vagi or cocainizing the mucous membrane of the trachea. An inspiratory air stream has no effect.

6. The Hering-Breuer reflex is present in the fowl in a modified form. The action of this reflex is chiefly to promote expiration.

7. Once begun expiration is continued by an expiratory reflex evoked by the passage of air out through the trachea.

8. Normally the expiratory phase of a respiratory movement lasts twice as long as the inspiratory phase.

9. These facts may be related to the differences in the direction of the flow of air during inspiration and expiration in the fowl.

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PROCEEDINGS

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Adrenal cortex and phosphorylation of vitamin B₁. By S. OCHOA and R. J. ROSSITER. *From the Department of Biochemistry, Oxford.*
(Received 28 October 1939)

Verzár and his colleagues [Verzár & McDougall, 1936, for references] have claimed that the phosphorylating capacity of an organism is impaired during adrenal cortical insufficiency. More specifically, Laszt [1938] suggests that the hormone of the adrenal cortex is essential for the formation of cocarboxylase (vitamin B₁ pyrophosphate) from vitamin B₁. All the evidence for such an hypothesis is of an indirect nature.

Using the method of Ochoa & Peters [1938] we have been unable:

(1) To detect any significant difference in the cocarboxylase content of boiled extracts of liver tissue taken from normal and adrenalectomized rats.

(2) To detect any significant difference in the increase of liver cocarboxylase following the injection (30 min. before killing) of 3 mg. free vitamin B₁ hydrochloride, i.e. in the ability of the liver of normal and adrenalectomized rats to phosphorylate vitamin B₁ *in vivo* (Table I).

TABLE I. Mean cocarboxylase content (expressed in $\mu\text{g./g.}$ fresh tissue) of liver of rats.

Treatment	No animals	Cocarboxylase	2 \times S.E.
Normal:			
No injection	7	1.3	± 0.4
Injected	4	11.1	± 2.2
Adrenalectomized:			
No injection	5	1.5	± 0.3
Injected	6	10.9	± 0.8

(3) To detect any difference in the *in vitro* synthesis of cocarboxylase by slices of liver tissue taken from normal and adrenalectomized rats (Table II).

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TABLE II. Cocarboxylase (expressed in $\mu\text{g./g.}$ fresh tissue) in slices of liver tissue after incubation for 30 min. at 38°C. Medium, Ringer-phosphate pH 8.4. Gas, oxygen.

Treatment	No additions	Plus 20 $\mu\text{g.}$ Vitamin B_1	Cocarboxylase synthesized
Normal	2.5	5.5	3.0
	3.6	11.0	7.4
Adrenalectomized	3.5	10.3	6.8
	2.5	4.4	1.9
	3.2	5.4	2.2

Points 1 and 2 have also been confirmed for kidney tissue (Table III). Here the synthesis of cocarboxylase from vitamin B_1 is less than in liver tissue, but again there is no difference in the phosphorylating capacity of kidneys from normal and adrenalectomized animals.

TABLE III. Cocarboxylase (expressed in $\mu\text{g./g.}$ fresh tissue) in kidney of rats.

Treatment	No injection	Injected
Normal	0.8	3.6
	1.2	3.9
	1.0	4.0
	1.0	5.8
Adrenalectomized	1.0	4.0
	0.8	5.8

The adrenalectomized animals were only used when they were in an *in extremis* condition produced by the adrenal cortical insufficiency. Such animals showed all the usual symptoms of the deficiency, e.g. extreme weakness, loss of appetite, loss of body weight, low body temperature. Similar animals not used for experiment died within eight days of reaching this stage.

Thus we are able to say that even in extreme adrenal cortical insufficiency, direct determinations of cocarboxylase offer no evidence for the theory that the adrenal cortical hormone is necessary for the phosphorylation of vitamin B_1 .

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The cholinergic nature of the nervous supply to the electrical organ of the *Torpedo* (*Torpedo marmorata*). By W. FELDBERG, A. FESSARD and D. NACHMANSOHN. *From the Maritime Station, Arcachon.* (Received 30 October 1939)

The electrical organ of the *Torpedo* has been compared with a collection of modified motor endplates which are not accompanied by muscle fibres. It appeared therefore possible that the nerves supplying this organ might act by liberation of acetylcholine. We have examined this possibility and the results obtained provide the experimental evidence for the acceptance of such a theory.

The finding of Auger & Fessard [1938] that the electrical organ is not excitable electrically after degeneration of its nerves made it difficult to imagine that the organ would be activated electrically by the impulses reaching the terminal network. Auger & Fessard therefore considered the possibility that the discharge might be brought about by liberation of some polarizing or depolarizing substance and the analogy of the electrical organ with the motor endplate suggested to Nachmansohn the possibility of a similar mechanism of nervous action at both structures. Having found a strong concentration of choline esterase in the motor endplates [Marnay & Nachmansohn, 1937, 1938; Nachmansohn, 1939], Marnay, on his suggestion, made a similar study of the electrical organ of the *Torpedo* and found a choline esterase concentration much stronger than that of any other organ hitherto examined [Marnay, 1937; Nachmansohn & Lederer, 1939].

Choline esterase content. We found for the electrical organ of the *Torpedo* a $Q_{\text{CH.E}}$ (mg. of acetylcholine hydrolysed in 1 hr. by 100 mg. of ground up tissue) of 170–250. In the *Raia* where the discharge from the electrical organ proceeds more slowly particularly with regard to its declining phase [Auger & Fessard, 1939] and resembles that obtained in the *Torpedo* after eserine, the $Q_{\text{CH.E}}$ was found to be 3–10.

Acetylcholine content. The electrical organ of the *Torpedo* contains a substance which could be identified as acetylcholine by its pharmacological reaction and its behaviour to alkali, choline esterase and eserine. We found 60–100 $\mu\text{g.}$ of acetylcholine per gram fresh tissue consisting of about 92% of water.

Liberation of acetylcholine. Part of the organ was perfused with eserinizied salt solution at a rate of 3–5 c.c./min. through the artery accompanying the second nerve to the organ. The venous effluent was

tested on the eserinizd leech muscle. For this purpose it had to be diluted on account of its strong salt concentration. In the absence of stimulation it had no action or caused only a weak contraction which disappeared as perfusion was continued. Perfusate collected during nervous stimulation caused strong contractions equivalent to those produced by solutions of acetylcholine of 1 : 15 millions to 1 : 40 millions. The identity of the active principle with acetylcholine was ascertained by the fact that it was destroyed by alkali and that it failed to appear in the venous effluent during nervous stimulation if no eserine had been added to the perfusion fluid.

Effect of eserine on the discharge. The addition of eserine to the perfusion fluid lengthened the single discharge. With repeated stimulation (frequency 10–15 per sec.) of the nerve fatigue set in much earlier than without eserine; the discharges diminished quickly and disappeared during a stimulation period of 3–5 min. Short lasting recovery took place when stimulation was discontinued for half a minute. Auger & Fessard [1940] have made similar observations on isolated prisms of the organ.

Effect of acetylcholine. Close range arterial injections of acetylcholine (10–200 μ g.) into the perfused organ connected with an amplifier caused potential changes in the same direction as those of the discharge. The electrical variation increased in voltage and in duration with the dose injected. The addition of eserine to the perfusion fluid lowered the threshold concentration of acetylcholine, effects being obtained with as little as 2.5 μ g. The responses to injected acetylcholine lasted a few seconds whereas the duration of the normally elicited discharge was a few milliseconds only. This difference can be explained by the fact that even a quick arterial injection cannot imitate the sudden and synchronous liberation of acetylcholine at the nerve endings and its subsequent quick destruction. In comparing the effects of arterial injections with those obtained on mammalian striated muscles we have also to take into account that in these the acetylcholine acts on the endplates but that the repetitive response is a reaction of the muscle fibres. These are absent in the electrical organ where the effect of acetylcholine in polarizing or depolarizing the membranes must be regarded as the final event, lacking any subsequent stimulating effect.

A full account of the experiments will appear in the *Archives Internationales de Physiologie*.

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The fixed framework of reticulocytes produced by injection of phenylhydrazine. By ERIC PONDER and SIDNEY VELICK. *From the Biological Laboratory, Cold Spring Harbour, Long Island, N.Y., and the Department of Protozoology, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Md. (Received 30 October 1939)*

The "fixed framework" of the mature erythrocyte of the rabbit has been found by Fricke, Parker & Ponder [1939] to be 2.2 g./100 c.c. of cells. This note deals with the fixed framework of reticulocytes produced in the rabbit by three intraperitoneal injections of 1 % phenylhydrazine hydrochloride in doses of 20 mg./kg. body weight.

The washed cells were haemolysed with twelve volumes of water and the electrical resistance and impermeability to haemoglobin restored by making the dilute suspension isotonic with NaCl, which also makes it possible to concentrate the ghosts by centrifugation at moderate speeds. The dry weights of the upper and lower layers of the centrifugate were determined, and the volume concentration of the ghosts in the lower layer calculated from the electrical resistance of the suspension and the suspending medium (in this case the supernatant fluid) by the equation

$$v = \frac{r_1/r_2 - 1}{r_1/r_2 + 1/X},$$

where r_1 is the resistance of the ghost suspension, r_2 the resistance of the medium, and $1/X$ the "form factor", which has been shown to be virtually the same for the "reversed" ghost as for the intact cell.

The ratio F of the haemoglobin concentration in the supernatant solution and the packed suspension was determined by direct comparison in the Pulfrich photometer at 4300 Å., from which c_1/c_2 , the ratio of haemoglobin concentration in the ghosts and suspending medium could be calculated from the equation

$$c_1/c_2 = (F - 1 - v)/v,$$

where c_1 and c_2 are the haemoglobin concentration in the ghosts and the suspension medium respectively. The haemoglobin concentration, c_2 , in the supernatant fluid was found to be equal to the dry weight corrected for the amount of NaCl present, which was 1% by weight both in the supernatant fluid and in the packed suspension.

The fixed framework is then given by the equation

$$\text{Fixed framework} = \frac{d_1 - (1-v) d_u - (c_1/c_2) c_2 v - av}{v} 100,$$

where d_u = g./c.c. upper fluid, d_1 = g./c.c. lower fluid, v = volume concentration of ghosts, a = g. NaCl/c.c. ghosts.

The values on normal cells, which confirm those obtained by Fricke *et al.*, is slightly less than the amount of lipid material and non-haemoglobin protein contained in the available analytical data:

% reticulocytes	0.3	0.0	50.0	48.0	42.0
Fixed framework g./100 c.c. cells	1.96	2.1	2.8	5.4	5.2
Fixed framework of reticulocytes	—	—	3.6	9.1	9.6

The values obtained on suspensions of cells containing many reticulocytes are 1.5–3 times as high as those obtained on cells from normal blood. If the difference is due entirely to the increased percentage of reticulocytes, then the fixed framework of reticulocytes is 2–4.5 times as great as that of mature cells. It must be kept in mind, however, that the phenylhydrazine cells contain an insoluble protein which has been designated as denatured globin, and which, though a non-integral part of the structure, would be included in the fixed framework determination if it does not diffuse out of the cell during haemolysis. This protein has never been observed in the normal reticulocyte. The presence of this protein, which would not be expected to be present in constant amounts, may explain the wide range observed in the fixed framework of the cells from phenylhydrazine-treated rabbits. The results show, however, that the amount of fixed framework of reticulocytes is considerably higher than that of the normal orthochromatic red cell.

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